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The Effects of Low Temperatures and Heparin on Potassium Exchangeability in Rat Diaphragm.

By

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The presence of more than one fraction of muscle potassium exchanging at different rates with tracer has been reported by several investigators. Most of the work has been confined to invertebrates and low vertebrates. Early work of this type has been reviewed by FENN (1936, 1940), and later by KROGH (1946).

Recently, the non-uniformity of potassium exchangeability in chick embryo muscle has been reported by WESSON, COHN and BRUES (1948), and in frog muscle by HARRIS (1952, 1953), STEINBACH (1954), CAREY and CONWAY (1954), CONWAY and CAREY (1955), HARRIS and STEINBACH (1956 a, b). However, KEYNES (1955) claimed full potassium exchangeability in frog muscles.

The few studies performed on mammalian muscles gave no satisfactory conclusions, concerning the possible presence of a "bound"[2] or "less freely exchangeable" fraction of potassium. While HEVESY and HAHN (1941) and MCLENNAN (1955) reported

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[2] Not necessarily chemically bound, but could be physically bound.

the existence of inexchangeable potassium fraction in skeletal muscles of the rat, CREESE (1951 a, 1954) and GOURLEY and JONAS (1954) reported complete exchangeability.

The present investigation which has been confined to *in vitro* studies and under steady state conditions demonstrates the existence of a bound fraction of potassium in rat diaphragm muscle at low temperatures. This fraction has been found to be temperature dependent.

In the course of studying[1] the effect of heparin on Rb^{86} loss from previously loaded muscles, results were obtained suggesting mobilization of muscle potassium by heparin. In this study direct evidence for the mobilization of the bound potassium by heparin has been obtained.

Methods.

Albino rats of both sexes weighing 120 g $\pm$ 5 (limits) were used for experimentation.

Solutions: Krebs saline (KREBS and HENSELEIT 1932) was used with diminished calcium content the composition being (mM): Na^+ 145, K^+ 5.9, Ca^{2+} 1.3, Mg^{2+} 1.2, Cl^- 126, HCO_3^- 25, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, and glucose 100 mg/100 ml. The saline was equilibrated with a gas mixture of 5 % CO_2, and 95 % O_2. This solution will be termed "inactive saline".

Radioactive salt and solutions: K^{42} was obtained spectroscopically pure in the form of carbonate from A.E.R.E., Harwell. The salt was neutralised to pH 8 with N HCl, and dist. water was added to provide a solution of potassium chloride of the same strength as that of the inactive saline. For loading muscles with K^{42}, 50 per cent of the potassium in the inactive saline was substituted by an equivalent amount of the radioactive potassium chloride solution. The solution thus prepared will be termed "radioactive saline".

For experiments with heparin, 1 mg/100 ml of heparin "Novo" in inactive and in radioactive salines were prepared. These solutions will be termed "heparinized inactive, and heparinized radioactive saline", respectively.

Apparatus and temperature control: The apparatus has been previously described by HASHISH (1954). It was slightly modified in that two containers were placed in the large thermoregulated ($\pm$ 0.5° C) reservoir so that two different effluents solutions could be led to the muscle chamber (effluent bath).

Muscle dissection: The animal was stunned and a hemidiaphragm muscle with its tendon and rib was removed into the dissecting bath

[1] HASHISH, MARRO and USSING (unpublished work).

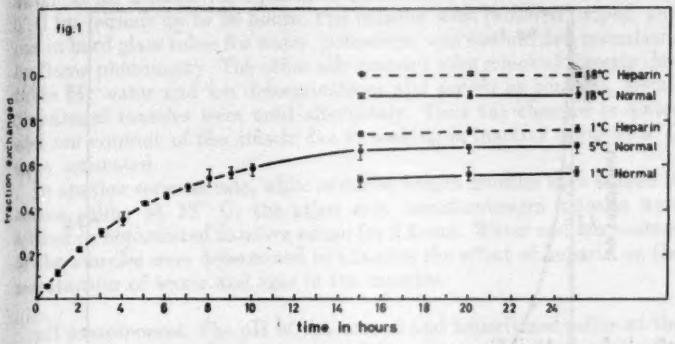

Fig. 1. Uptake of K^{42} by rat diaphragm: The ordinate shows the fraction of K exchanged, given by the ratio: activity per mg K^+ in muscle/activity per mg K^+ in Krebs saline. The abscissa shows the periods of muscle soaking in active solution. The interrupted curves are for heparinized solutions and the continuous ones for normal solutions. The temperatures of the experiments are indicated. Each point on the curves represents a mean of three experiments with maximum and minimum values.

containing oxygenated inactive saline at 38° C, for further dissection and trimming, thus avoiding potassium loss by anoxia (CREESE 1954). The other half of the diaphragm muscle served as control.

The entry of K^{42}: The muscles after dissection were attached to simple glass holders, and soaked in baths containing inactive saline at 38° C, for 2 hours, for equilibration and recovery from trauma of dissection. They were then removed into other soaking baths containing inactive saline at the desired low temperature for one hour for equilibration, followed by radioactive saline at the same temperature. The muscles were removed at intervals indicated in Fig. 1, the rib cut away and the adhering saline on the surface wiped off on a dry porcelain tile. They were then placed in hard glass tubes (10 ml capacity) for weighing. The loss in weight after overnight heating to 110° C gave the water content. 0.1 ml conc. nitric acid plus 0.1 ml water were added and, after heating in a water bath for one hour, about 5 ml water was added and the heating continued for a further hour. The muscle thus dissolved except for a small pellet of fat. The contents were diluted to 25 ml in a graduated flask to give a solution suitable for radioassay and flame photometry. At intervals, one ml of the soaking fluid was drawn, diluted to 25 ml and the specific activity was measured. The influx experiments were carried out by soaking a set of muscles in radioactive saline and heparinized radioactive saline at 1°, 5°, or 18° C. The muscles were adequately aerated by the oxygen-carbon dioxide mixture.

The exit of K^{42}: The muscle after 2 hours equilibration in inactive saline at 38° C was soaked in radioactive saline at 38° C for 3—4 hours

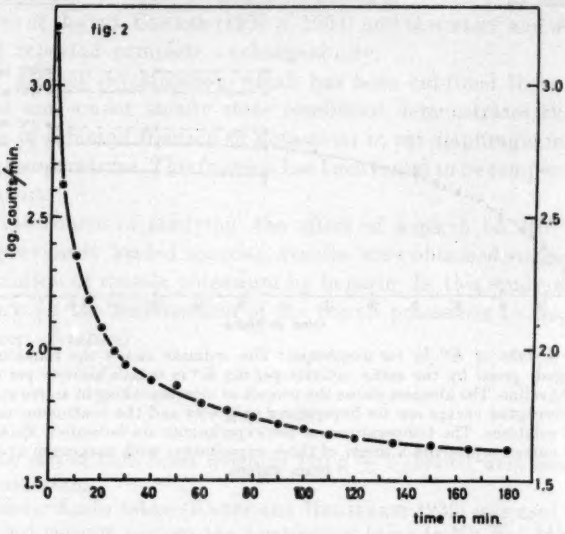

Fig. 2. Semilog plot, of the K^{42} loss from loaded diaphragm muscle into inactive Krebs saline at 5° C, as carried out by the effluent bath method. The ordinate shows the corrected log counts/min of the saline collected. The abscissa shows the periods during which the collections were made.

to ensure complete exchangeability of muscle potassium, (CREESE 1954). It was transferred into a soaking bath containing radioactive saline at 5° C for one hour for equilibration. Cold (5° C) inactive saline was allowed to flow — from the 2 litre bath situated in the large reservoir — through the inflow tube into the effluent bath at rate of about 10 ml/min. The overflow was siphoned down to waste. The loaded muscle was then immersed in inactive saline bath at 5° C for 1/2 min to remove radioactivity adhering on the muscle and holder. It was put in the effluent bath and the siphoned saline was collected during one min intervals in 10 ml capacity graduated glass tubes, for a period of three hours. The intervals during which the effluent was collected are indicated in Fig. 2.

All the baths were continuously aerated by the gas mixture. The limiting factors for steady washout collections were adequate muscle oxygenation and constant saline inflow. Excessive oxygenation caused temporary blockage of the siphon tube. The volume of the saline collected in the tube was made up to 10 ml. The solutions were thus ready for radioassay.

Potassium, sodium and water determinations. In a series of rats, hemidiaphragm muscles of one side were dissected, soaked in inactive saline

at 38° C for 2 hours for equilibration, transferred to inactive saline at 5° C for periods up to 10 hours. The muscles were removed, wiped, and put in hard glass tubes for water, potassium, and sodium determinations by flame photometry. The other side muscles were removed directly into tubes for water and ion determinations and served as controls. Hemidiaphragm muscles were used alternately. Thus the changes in water and ion content of the muscle due to soaking in inactive saline at 5° C were estimated.

In another series of rats, while hemidiaphragm muscles were soaked in active saline at 18° C, the other side hemidiaphragm muscles were soaked in heparinized inactive saline for 3 hours. Water and ion content of the muscles were determined to examine the effect of heparin on the net transfer of water and ions in the muscles.

pH measurement. The pH of the normal and heparinized saline at the working temperatures was measured by means of a glass electrode. Addition of heparin or lowering the temperature did not detectably change the pH.

Results.

The uptake of K^{42}: While it has been reported by CREESE (1954) that all muscle potassium is exchangeable at 38° C, only 55 % of muscle potassium is exchangeable at 1° C, 68 % at 5° C, and 91 % at 18° C (Fig. 1).

When heparin was added the exchangeable potassium fractions at 1° C and 18° C were 75 % and 100 % respectively. Thus at 1° C, heparin has mobilized 20 % of the total or 44 % of the bound potassium and at 18° C it has mobilized 9 % of the total or all the bound potassium.

For the K^{42} entry at 5° C, the process does not appear to be a simple exponential one. The measured "apparent" half-life of K^{42} entry, which does not represent true half-life for reasons discussed later (p. 195), is gradually increasing from 0.75 hour after 0.5 hours soaking to about 19 hours after 10 hours soaking. 50 % of the muscle potassium exchanges in 7 hours and equilibrium is achieved after 15 hours.

The leakage of K^{42}: Fig. 2 shows the rate of outward movement of K^{42} from labelled muscle washed with inactive saline at 5° C. The steep decline of the curve during the first hour is due mainly to the radioactivity in the rib (HASHISH 1954) and to the loss of extracellular potassium (HARRIS and BURN 1949). The process is not a simple exponential one. The apparent half-life of K^{42} loss in-

Table I.

Water, potassium, and sodium content of:
A. Hemidiaphragm muscles freshly removed or soaked in normal inactive saline at 5° C for 10 hours.
B. Hemidiaphragm muscles soaked in normal inactive or heparinized inactive saline at 18° C for 3 hours.

S. D. = Standard Deviation. n = number of experiments.

		S. D.	n
Per cent Water Content			
A. Fresh	78.3	1.3	(17)
Soaked	79.2	1.1	(17)
B. Normal	78.8	1.2	(6)
Heparinized	78.6	1.4	(6)
mmoles K/kg Muscle			
A. Fresh	90.3	7.9	(18)
Soaked	78.9	7.5	(14)
B. Normal	79.7	8.1	(6)
Heparinized	80.1	6.8	(6)
mmoles Na/kg Muscle			
A. Fresh	37.1	5.8	(18)
Soaked	80.6	17.3	(15)
B. Normal	72.4	11.5	(6)
Heparinized	68.2	14.3	(6)

creases gradually from about one hour during the 1st—2nd hour period to about 3 hours during the 2nd—3rd hour period of washing.

The corresponding figures for the apparent half-lives calculated from the entry curve at 5° C are of the same order of magnitude.

Effect of cooling on water and ion content of the muscle: Soaking the muscles in inactive saline at 38° C caused a loss of 7 to 12 % in muscle potassium and a gain of 70 to 80 % and of 1.2 to 1.6 % in muscle sodium and water respectively (Hashish 1954, Creese 1954). The corresponding values for muscles soaked for 10 hours at 5° are shown in Table I A. Cooling caused a rise of about 1 % in water, 118 % in sodium and a fall of about 14 % in potassium content of the muscles. The distribution of the ion content in the muscles is normal. Accordingly, cooling the inactive saline from 38° C to 5° C caused an extra loss of about 4 % of muscle potassium and an extra gain of about 37 % of muscle sodium. Heparin caused no detectable net transfer of ion nor of water as shown in Table I B.

Discussion.

The rat diaphragm muscle provides a suitable tissue for *in vitro* studies. DALE and GADDUM (1930), and BÜLBRING (1946) popularised its use. The muscle is thin being 0.6 mm in thickness for rats weighing 120 g (CREESE 1954, CREESE, HASHISH and SCHOLES 1958), and can be adequately oxygenated (HILL 1928). The suitability of the Krebs saline as a bathing fluid for the muscle has been discussed by HASHISH (1954) and CREESE (1954).

The exchange studies in the present work were made without the complications of net transfer. After the equilibration period in the saline, potassium, sodium, and water content of the muscles remained unaltered for several hours. The pH also was tolerably constant around 7.4 (CONWAY 1950, CREESE 1951 b, 1954).

Lowering the temperature of the bathing fluid would be expected to impede the "sodium pump" mechanism described by DEAN (1941), thus resulting in sodium gain and subsequent potassium loss in the muscle. A fall of a few millivolts in the resting potential of the diaphragm muscle soaked in Krebs saline at 1° C reported by CREESE (personal communication) is in accordance with the estimated ionic changes. The net ionic changes, which were over in one hour, indicate the passage of the muscle to a steady state, and suggest that the process is reversible. The existence of a steady state favours quantitative measurements of ionic fluxes. The turnover rates for potassium entry and exit were found at 5° C to be of the same order during corresponding periods of muscle soaking.

The entry of K^{42} into the muscle at low temperature is not a first order process, the calculated half-life increases progressively denoting non-uniformity of potassium exchange. The half-life of the turnover rate thus measured does not represent a true half-life. It has to be corrected for interfibre diffusion delay (HARRIS and BURN 1949, KEYNES 1954). The entry during the first hour is enhanced by the quick uptake of K^{42} by the rib and the extracellular space (HASHISH 1954). The corrected values for the half-life in diaphragm muscles of 0.6 mm thickness are 2.5 times smaller than the measured ones (CREESE 1954, HARRIS 1956). The kinetics of K^{42} loss from the muscle is subject to similar considerations. The steeper fall in the curve during the first hour is mainly due to the contribution of the rib to the measured activity, and partly to the extracellular potassium leakage.

The similarity of the measured half-lives during identical periods of entry and exit shows that an equilibrium has been attained.

That the measured rates of turnover at low temperature are slow as compared with those at 38° C, (HASHISH 1954, CREESE 1954) indicate that the exchange process is temperature dependent. The influence of temperature is thought by HARRIS (1952) to be twofold; it causes mobilization of internal potassium, and enhances the rate of transfer across the cell boundary.

It is customary to obtain a curve semilog line rather than a straight one for K^{42} loss from muscles (HASHISH 1954, CREESE 1954, HARRIS 1956, KEYNES and LEWIS 1951). This is expected since one is dealing with an assembly of fibres of different diameters. The presence of more than one fraction of potassium exchanging at different rates is indeed a major contributing factor. Moreover, the interfibre diffusion delay caused by a comparatively large extracellar space (26 ml/100 g muscle) (CREESE and HASHISH 1953) is also important. The possibility of the different fibres containing different ionic concentrations, or having different permeabilities cannot be overruled. CREESE, NEIL and STEPHENSON (1956) gave a valuable mathematical discussion of the problem.

In this study the presence of a bound form of potassium in the muscle has been demonstrated. The tendency of the bound potassium fraction to diminish by raising the temperature explains the failure of investigators to detect it in mammalian muscle at body temperature (NOONAN, FENN and HAEGE 1941, CREESE 1951, 1954, GOURLEY and JONAS 1954). At 20° C MCLENNAN (1955) demonstrated an "apparently inexchangeable" fraction of potassium in sartorius and extensor digitorum muscle of the rat. The work on amphibian muscles at low temperatures revealed the existence of an inexchangeable or bound potassium fraction (HARRIS 1952, 1953).

It may not be absolutely correct to speak of bound potassium when the K^{42} entry study is confined to a limited period. The so called bound potassium fraction may well prove to be "a very slow, inconveniently measureable" but exchangeable fraction. The entry curves in the present study at 1° and 5° C favour this nomenclature, since they tend to rise slightly rather than become absolutely horizontal. It is however permissible to label this apparently inexchangeable fraction of potassium as bound bearing in mind the possibility suggested. Long soaking periods would settle the argument.

The incomplete and non-uniform exchangeability of potassium in muscle indicates the presence of different fractions of potassium, one of which is unionised or slightly ionisable at low temperature. The different potassium fractions presumably exchange at different rates with extracellular ions, meanwhile they are interchangeable. The overall measured rate of turnover accordingly does not represent a true flux but is a function of all the rates involved. At higher temperatures the rate of exchange among the different potassium fractions is so increased that all behave as one "fraction". This suggestion agrees with the explanations of possible mechanisms for potassium turnover in amphibian muscle given by HARRIS (1952).

That the bound potassium has been mobilized when heparin is added or the temperature is raised suggest that their modes of action may be analogous.

Summary.

1. The K^{42} entry into, and leakage from diaphragm muscles of the rat *in vitro* have been studied at low temperatures. The two processes are not of the first order type.

2. The study revealed the existence of a substantial fraction of muscle potassium as a bound form.

3. The bound potassium fraction was mobilized when the temperature was raised or heparin was added.

4. Cooling the muscle to 5° C caused a net potassium loss and sodium gain.

5. Heparin had no effect on the water, potassium and sodium content of the muscle.

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A Method for Prolonged Intra-Arterial or Intra-Venous Infusion in the Unrestrained Animal.

By

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In previously described methods for infusion in laboratory animals the infusion device has been separated from the animal. The movements of the animal have been limited by the length of the tube from the infusion device. To minimize the risk of twisting the tube, the movements of the animal have been restricted by the use of a small cage (WOERNER 1938), by tying the animal (KINASH and HAIST 1954) or even by sectioning the sciatic and femoral nerves (BARRON and STATE 1949). In the method which will now be described, the animal carries the infusion device, which is refilled once or twice daily, with it. In this way the risk of twisting the tube is eliminated and the movements of the animal are very little restricted.

The apparatus (Fig. 1C), which is attached to the animal with a harness made of metal net covered with plastic foam, is mainly made of plexi-glass. It consists of a container, which, when the apparatus is intended for dogs, has a volume of 2.5 litres. In this, there is a plastic bag for the fluid to be infused.[1] The capacity of this bag is a fifth of the volume of the container. Through the wall of the container the bag has an inflow and an outflow. The pressure of the air in the container surrounding the plastic bag can be elevated by pumping air into the container through an ordinary

[1] Manufactured by Fenwal Laboratories, Inc., Framingham, Massachusetts, U.S.A.

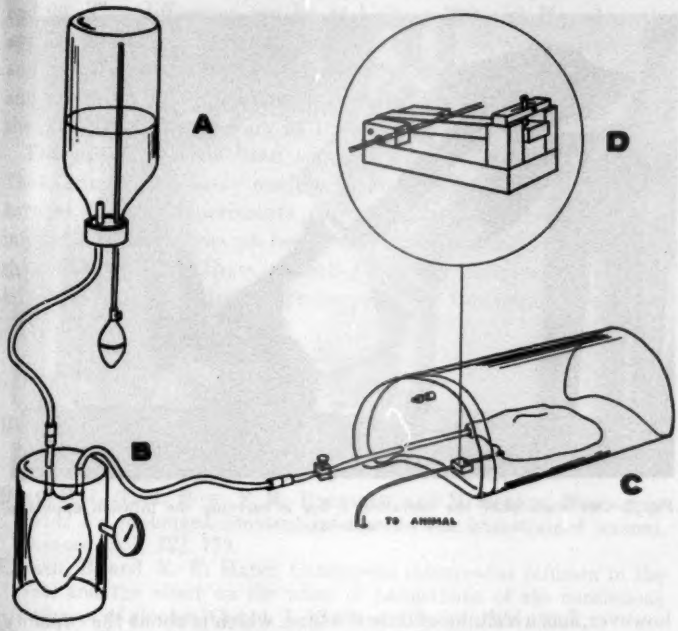

Fig. 1 The infusion apparatus (C) is for the refiling once or twice daily, via a "pressure measurement device" (B), connected to a standard transfusion set (A), from which the fluid is pumped into the apparatus.

bicycle-tire-valve. In this way the fluid is pressed through the outflow which, via a filter, is connected to a slim polyethylene catheter. The flow through the catheter can be regulated by compressing it (Fig. 1D). Covered by a thick plastic tube, the catheter goes in a sling, until, somewhere on the back, protected by the harness, it goes through the skin of the animal. With the help of a stylet it is pulled from here subcutaneously to the vessel into which the fluid is to be infused.

The apparatus is refilled from a standard infusion bottle into which air is pumped (Fig. 1A). The fluid goes through the tube of a standard transfusion set, but before it reaches the apparatus, it passes a "pressure measurement device" which is connected to the apparatus only during the refilling (Fig. 1B). The "pressure measurement device" consists, just as does the apparatus, of a plexi-glass container with a plastic bag inside. This container,

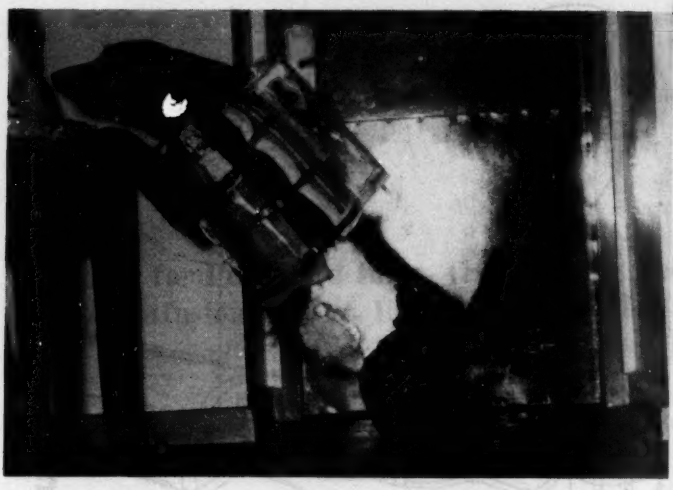

Fig. 2. One week after the operation a dog is carrying the infusion apparatus with ease.

however, has a volume of only 0.2 litre, which is about the capacity of the plastic bag. The fluid passes the bag on its way to the apparatus, and the more it expands the bag the more the air surrounding it is compressed, and this compression can be measured on a manometer. When the refilling is completed, the tube from the bottle to the "pressure measurement device" is clamped. The manometer then shows the pressure in the apparatus, as there is a free communication between the two plastic bags.

The pressure in the apparatus decreases as the fluid is infused. In accordance with Boyle's law the pressure, when the plastic bag is empty, is four fifths of what it was when the bag was filled. Thus, if the pressure is 300 mm Hg when the apparatus has just been refilled, it will never go below 240 mm Hg if the temperature is unaltered. This pressure is well above the arterial pressure of a normal animal, which means that there is no risk of blood coming into the catheter and clotting there, even if the plastic bag should be completely emptied.

The flow from the apparatus into the blood stream has proved to be proportional to the difference between the pressure in the apparatus and the pressure in the vessel where the catheter is

ending. This difference can be as high as 300 mm Hg when the apparatus has just been refilled and the blood pressure is zero, and as low as 150 mm Hg when the apparatus is practically empty and the mean blood pressure 90 mm Hg. Thus with this apparatus the infusion flow may vary as 1 : 2.

The apparatus has been successfully used on dogs (Fig. 2). These animals are easily made to accept the unpleasantness of the harness. In the experiments performed, the uterine artery was infused and there was no need continuing this infusion for more than three weeks. There seemed to be no technical difficulties, however, for a considerable prolongation of this period.

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The Synaptic Linkage of 'Direct' Inhibition.

By

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LLOYD (1941) originally proposed that the inhibitory action of group I impulses onto motoneurones of antagonist muscles was exerted monosynaptically and hence it was called direct inhibition. This conclusion was based on the finding that inhibition of a monosynaptic reflex occurred without measurable latency *i. e.* it was found to persist until the interval between the inhibitory and excitatory volleys was made vanishingly small (LLOYD 1941, 1946, LAPORTE and LLOYD 1952). However, it was pointed out firstly by RENSHAW (1942) and later in more detail by ECCLES, FATT and LANDGREN (1956) that by this method the latency of the inhibitory action is measured only in respect of the time of initiation of the testing reflex discharge and that therefore the central latency for direct inhibition must be longer than for the monosynaptic excitatory action.

Using intracellular recording BROCK, COOMBS and ECCLES (1952) discovered that direct inhibition was caused by a hyperpolarization, the inhibitory postsynaptic potential (IPSP). The latency relative to the incoming volley was about 1.3 msec, which is 0.8 msec longer than for the monosynaptically evoked excitatory postsynaptic potential (EPSP). The long latency for the IPSP was originally taken to indicate either that the intramedullary conduction time was long or, alternatively, that the synaptic delay was longer at inhibitory synapses. However, ECCLES, FATT and LANDGREN (1956) on the basis of new experimental evidence discarded these explanations and put forward the hypothesis that

an interneurone was interpolated in the inhibitory pathway and that transmission between the primary afferents and the inhibitory interneurone was responsible for the long latency found for the IPSP. Thus 'direct' inhibition would be due to a disynaptic pathway. Evidence was presented that the interneurone on the pathway was located in the intermediate nucleus of Cajal.

The hypothesis that an interneurone is interpolated in the direct inhibitory pathway has not been universally accepted. WILSON and LLOYD (1956) as well as GRUNDFEST (1957) still favour the monosynaptic alternative. Previously one finding has been reported, which perhaps could more easily be interpreted if inhibition actually were exerted by monosynaptic action. LLOYD (1943) found that the curves for the relationship between the size of the afferent volley and the monosynaptic facilitatory action on one hand and the reciprocal inhibition on the other were identical. This finding would be compatible with disynaptic transmission only if the synaptic activation of the interneurones was so effective that even single or a few afferent impulses caused interneuronal discharge. However, with all systems of cells investigated in the central nervous system, excitation is known to require appreciable summation. This holds true even for sensory pathways where the synaptic linkage is much stronger than with motoneurones (cf. BISHOP 1953, HOLMQVIST, LUNDBERG and OSCARSSON 1956, AMASSIAN and DE VITO 1956). ECCLES *et al.* (1956) showed that some summation was required for excitation of the interneurone that was assumed to mediate I a inhibition.

In the present paper it will be demonstrated that spatial facilitation does occur in the I a inhibitory pathway. Four different methods have been applied to test the hypothesis of an interneurone in the I a inhibitory pathway.

(a) Comparison of the excitatory and inhibitory effects produced by the same I a fibres with all gradations of I a volley size;
(b) Evidence of facilitation in the I a inhibitory pathway to the motoneurone;
(c) The effect of post-tetanic potentiation on transmission in the inhibitory pathway;
(d) The occurrence of occlusion in the I a inhibitory pathway.

A preliminary report of some of the results has been given (ECCLES and LUNDBERG 1957).

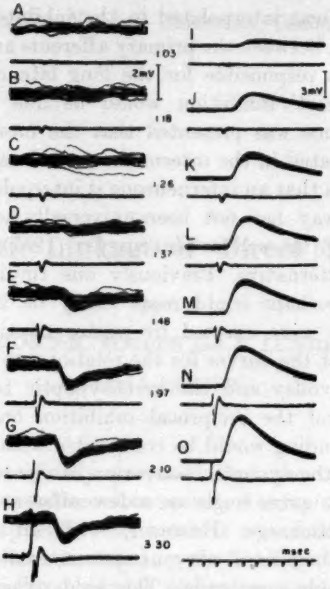

Fig. 1. The upper traces are intracellular records from a posterior-biceps-semitendinous motoneurone A—H and from a quadriceps motoneurone (I—O), internal positivity (depolarization) being signalled upwards. Observe that in the triphasic records from the L VI dorsal root entry zone, negativity unconventionally is signalled by a downwards deflection. The two sets of records were obtained at 10 minutes' interval, on stimulation of the quadriceps nerve. Stimulus strengths relative to threshold are indicated between the corresponding records. All intracellular records are at the same speed, A—H at the amplification on the left, I—D at the amplification on the right.

Methods.

The experiments were performed on cats under light anaesthesia from pentobarbital sodium and the spinal cord was sectioned in the upper lumbar region. The technique used for intracellular recording has been described by Brock, Coombs and Eccles (1952). In all experiments recording was made with micro-electrodes filled with a solution of 0.6 *M* K_2SO_4. All records are obtained by superposition of about 10—20 faint traces.

Results.

In Fig. 1 the establishment of the direct IPSP and of the monosynaptic EPSP can be compared. Both series of records were obtained on stimulation of the same quadriceps nerve, which dis-

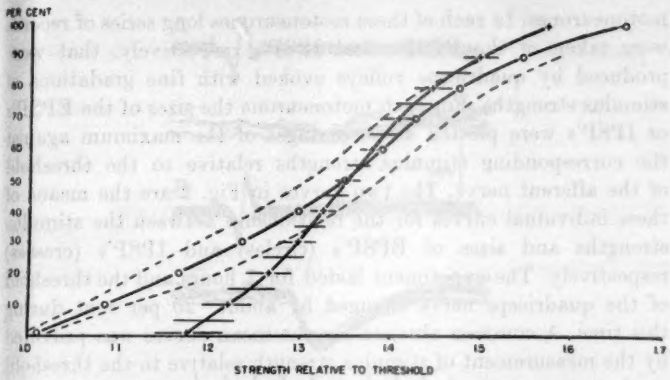

Fig. 2. The curves show the relationship between the stimulus strength and the height of the EPSP's (circles) and the IPSP's (crosses). Recording was made from 13 posterior-biceps-semitendinous cells and from 13 quadriceps cells in one experiment. The quadriceps nerve was stimulated with fine gradations of strength. For each neurone the size of the EPSP's or IPSP's were plotted against stimulus strength and the curves in the figure are means of these EPSP or IPSP curves from the individual neurones. The standard deviation from the mean EPSP curve is given by the hatched lines and the standard deviation from the mean IPSP curves by the horizontal bars. For maximal activation of group I a a stimulus strength of 1.7 times threshold was required.

played the separation into I a and I b components (H) (cf. BRADLEY and ECCLES 1953, LAPORTE and BESSOU 1957, ECCLES, ECCLES and LUNDBERG 1957). The left series (A—H) show intracellular records from a motoneurone of the posterior biceps-semitendinosus nucleus and demonstrate the growth of the IPSP with increasing I a volleys (A—G). The corresponding records in the right series were obtained from a quadriceps motoneurone some ten minutes after the previous series, and show the monosynaptic EPSP's evoked by identical I a volleys from the quadriceps nerve. The IPSP is not observable until the stimulus strength has been raised to 1.37 times threshold, giving the sizable I a volley observed in the dorsal root record in D. On the other hand the EPSP appears with the minimal visible dorsal root volley in I and has attained approximately half maximal size at the stimulus strength of 1.37 in L.

This difference between the relationship of IPSP's and EPSP's is further illustrated by the composite diagram of Fig. 2 which is constructed from the pooled data derived from intracellular records of 13 quadriceps and 13 posterior biceps-semitendinosus

motoneurones. In each of these motoneurones long series of records were taken of the EPSP's and IPSP's respectively, that were produced by quadriceps volleys evoked with fine gradations of stimulus strengths. For each motoneurone the sizes of the EPSP's or IPSP's were plotted as percentages of the maximum against the corresponding stimulus strengths relative to the threshold of the afferent nerve. The two curves in Fig. 2 are the means of these individual curves for the relationship between the stimulus strengths and sizes of EPSP's (circles) and IPSP's (crosses) respectively. The experiment lasted for 7 hours and the threshold of the quadriceps nerve changed by about 20 per cent during this time. A common abscissa for the mean curves was provided by the measurement of stimulus strength relative to the threshold at that time. A check of the validity of this procedure was provided by the finding that the curves for the growth of the I a volley with increase of stimulus strength remained virtually unchanged throughout the experiment.

The hatched lines in Fig. 2 give the limits of variation by one standard deviation from the mean EPSP curve, and similarly the horizontal bars from the mean IPSP curve. Both standard deviations were calculated for the 19 percentile values indicated by the horizontal bars of the IPSP curve.

The curves in Fig. 2 display many noteworthy features. The EPSP appears at threshold for stimulation as has already been illustrated for the monosynaptically evoked EPSP (Eccles, Eccles and Lundberg 1957, Fig. 3, lower graph, where the EPSP attains its maximum before the threshold of group I b fibres is reached). The IPSP, on the other hand, did not appear until the stimulus strength was raised to 1.17 times threshold, at which strength the I a volley had attained about 20 per cent of its maximum size. On the other hand, in the medium range of stimulation, the IPSP curve slopes more than the EPSP curve and actually crosses it to reach a maximum before the maximum of I a volley. The standard deviation of the two curves display the same characteristic differences. For the IPSP curve the maximum standard deviation is found at the threshold for the appearance of inhibition, whereas the EPSP curve displays its minimal standard deviation at threshold stimulation. At higher intensities of stimulation these relations are inverted.

If excitation of interneurones were a necessary requisite for the establishment of IPSP, and if these interneurones required some

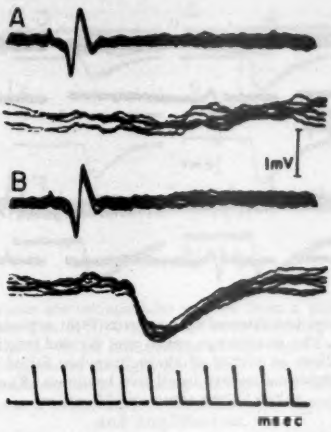

Fig. 3. The lower traces are intracellular records from a sartorius motoneurone. The adductor femoris nerve was stimulated at a strength about three times higher than that giving maximal group I activation. A was taken before and B after stimulation of the adductor femoris nerve at 500/sec during 15 seconds. Each record is composed of about 10 superimposed traces the frequency of stimulation being 1/sec. Both intracellular traces at the same amplification.

summation for excitation, we would expect that a low threshold volley would be ineffective in evoking an IPSP in the majority of the motoneurones. If we assume that each of these interneurones receives a limited convergence of I a afferents, the threshold for the minimal observable IPSP would be expected to vary accordingly as the interneurones evoking this threshold IPSP are supplied mainly with low or high threshold I a afferents. Finally, the production of a maximum IPSP by a submaximal I a volley is explicable if all of the inhibitory interneurones for that motoneurone were activated by a submaximal I a volley.

Occasionally inhibitory curves were recorded which were outside the range of the typical IPSP curve of Fig. 2. In the intracellular records A and E in Fig. 5 IPSP's were evoked by very weak volleys in the nerves from rectus and vasto-crureus respectively. Presumably some of the inhibitory interneurones supplying this p. biceps-semitendinosus motoneurone received a number of I a afferents in the lowest threshold range.

Another extreme is represented by motoneurones in which IPSP's can be evoked only by impulses in the highest threshold I a afferents, or when the maximal I a volley is without any action

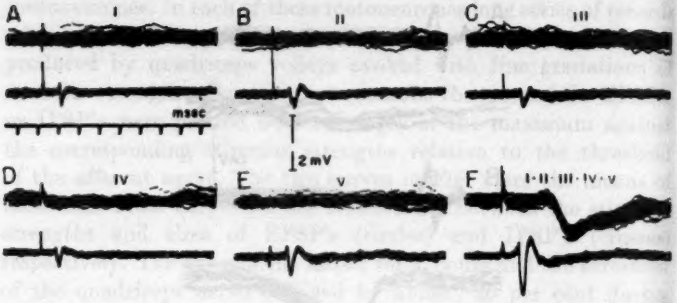

Fig. 4. The upper traces are intracellular records from a posterior-biceps-semitendinosus motoneurone. The quadriceps nerve was divided into five branches (T—V) Maximal Group I volleys in either of these branches failed to evoke any IPSP (A—E), whereas synchronous volleys in all five branches (F) evoked a large IPSP, taken at the same amplification.

on a motoneurone belonging to an antagonist muscle. In Fig. 3 A a maximal group I volley in the nerve to adductor femoris did not evoke any IPSP in a motoneurone to sartorius. Some seconds after a conditioning tetanization of the adductor femoris nerve for 15 seconds at 500/sec a testing volley in the same nerve produced an IPSP (B). This cannot be attributed to a post-tetanic potentiation at the inhibitory synapses on the motoneurone, since prior to tetanization, the same volley was completely ineffective. Therefore the potentiation must occur at some synapse upstream in the inhibitory pathway, which would be the postulated synaptic connection between the primary I a afferents and the inhibitory interneurones.

The importance of the above experiment is that, even if impulses in primary afferent fibres are ineffective in establishing an IPSP on a motoneurone, these fibres may, nevertheless, be an essential component of the inhibitory pathway. This conclusion is confirmed by the intracellular records from a posterior biceps-semitendinosus motoneurone in Fig. 4. The quadriceps nerve was split into five parts and maximal group I volleys in each of these branches did not evoke any trace of IPSP (A—E). When all the five branches were synchronously stimulated the large IPSP in F was recorded. This effect could not be attributed to some chance variation of the membrane potential to equivalence with the equilibrium potential for the IPSP (cf. Coombs, Eccles and Fatt 1955) during the traces A to E, because it was repeatedly

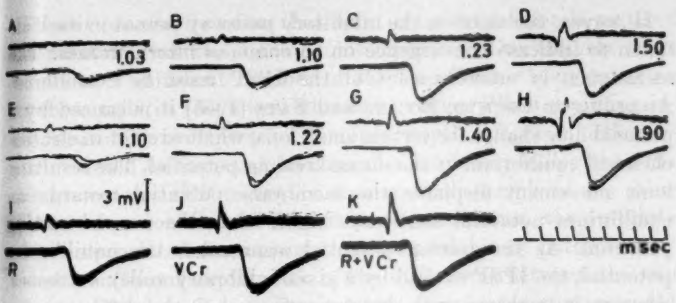

Fig. 5. The lower traces are intracellular records from a posterior-biceps-semitendinosus motoneurone. The IPSP's in A—D were recorded on stimulation of the rectus nerve, those in E—H on stimulation of the vasto-crureus nerve. Stimulus strengths relative to threshold strength are indicated on the records. Records I—K were obtained successively and K shows occlusion of IPSP's on simultaneous stimulation of rectus and vasto-crureus. All intracellular records at the same speed and amplification.

obtained during random sequences of the observations A to E.

The occurrence of spatial facilitation in a synaptic system is only one consequence of the principle of convergence. In other circumstances when cells can be excited from different presynaptic sources, convergence may lead to occlusion. If this occurred at the inhibitory interneurone, the IPSP evoked in a motoneurone on synchronous stimulation of two nerves would be smaller than the algebraic sum of the IPSP's resulting on individual stimulation of these two nerves. Nevertheless, a certain small reduction in the size of the combined IPSP's would be expected due to the approach to the equilibrium potential (see below). This occlusion is illustrated in the intracellular records from a posterior biceps-semitendinosus motoneurone (Fig. 5). Large IPSP's were evoked in this neurone by I a volleys from the nerve to rectus (A—C) and also from vasto-crureus (E—G). After the series partly illustrated in A—H had been taken, the maximum IPSP from rectus (I) and vasto-crureus (J) were recorded, whereupon record K was obtained with synchronous stimulation of the two nerves. A subsequent control demonstrated that the effect from rectus or vasto-crureus alone had not changed. The occlusion was nearly complete. The size of the IPSP from rectus in I was 70 per cent of that evoked by the vasto-crureus volley in J, but, on synchronous stimulation, the IPSP was only a few per cent larger than that resulting from stimulation of the vasto-crureus alone.

However, occlusion in the inhibitory pathway cannot in itself be taken to indicate convergence on to common interneurones. The mechanism of establishment of the IPSP must be considered. According to COOMBS, ECCLES and FATT (1955) it is caused by a permeability change for certain small ions, which are not in electrochemical equilibrium at the normal resting potential. The resulting ionic movement displaces the membrane potential towards an equilibrium potential somewhat higher than the normal resting potential. As the resting potential approaches the equilibrium potential, the IPSP evoked by a given inhibitory volley decreases, whereas it increases with depolarization when the difference between the resting potential and the equilibrium potential increases. It is therefore obvious that, if IPSP's are superimposed, the effect will be less than additive. It is, however, extremely unlikely that this mechanism is the sole cause of the almost complete occlusion in Fig. 5. The equilibrium potential for the IPSP is usually about 80 mV (COOMBS *et al.* 1955), whereas the resting potential of the cell in Fig. 5 was 60 mV. Since the IPSP in K was not larger than 4 mV, the safety margin would seem to be sufficient. Undoubtedly it would be possible to exclude this possibility more directly, for example by superimposing the I a IPSP on IPSP's from other sources (*i. e.* I b, II, III or Renshaw) or by comparing the IPSP sizes after shifting the membrane potential by a current applied intracellularly. Unfortunately, the cell in Fig. 5 was lost before this could be done and such a high degree of occlusion was found very rarely. For the time being we must confine ourselves to the statement that the effect in Fig. 5 as well as the upper end of the IPSP curve in Fig. 2 very strongly suggest occlusion at an interneuronal level.

Discussion.

The present experiments have demonstrated beyond doubt that spatial facilitation does occur in the direct inhibitory pathway. Presumably several factors contribute to the differences between the present results and those of LLOYD (1943). LLOYD used high-spinal unanaesthetized cats, whereas in our experiments, the excitability of the cells would be somewhat depressed by the narcotic. Even in cats, anaesthetized by pentobarbital sodium some motoneurones have been found in which the inhibitory potential does appear with a very small dorsal root volley. This

does not necessarily mean that little or no summation is required for excitation of the assumed interneurone. Alternatively there would be a sufficient number of the I a afferents converging on to these cells in the lowest threshold range. When assessing inhibitory action by depression of monosynaptic reflexes, an effective action would be expected for a smaller inhibitory volley than the one giving an IPSP in any particular small sample of the same species of motoneurones. With stronger stimulation, the postulate of an interpolated neurone on the direct inhibitory pathway provided a sufficient explanation of the differences between the two curves plotted in Fig. 2. Presumably, comparable curves would be given when virtually pure I a afferent volleys are employed for inhibition of testing reflexes, though this has not yet been done. It must be recalled that, in LLOYD's early experiments (1941, 1943) dorsal roots were stimulated and that consequently the actual afferent volley which was used as an index for comparison of monosynaptic and direct inhibitory effects included impulses in many other fibres besides those giving rise to direct inhibition. An unknown degree of distortion was thereby introduced into the curves.

It has been pointed out throughout the previous section that the spatial facilitation in the direct inhibitory pathway is sufficiently explained by the interneurone assumed to be interpolated on that pathway. The question may now be asked: Is there any explanation which would be compatible with a monosynaptic linkage? It might appear that a possible explanation derives from the recent demonstration by KATZ and THESLEFF (1957) of a non-linear dose-response relationship at the neuro-muscular junction. When small doses of acetylcholine were applied from two barrels, simultaneous application often produced more than a simple additive effect. With small doses the relation between dose and depolarization had an S-shaped rather than a linear start. If it be assumed that with the hypothetical inhibitory transmitter the dose-response curves were S-shaped even to a more exaggerated degree than found for acetylcholine at the endplate, would it not then be possible to account for the spatial facilitation in the inhibitory pathway as a phenomenon compatible with a monosynaptic linkage of the I a inhibition? However, this explanation can be rejected, because, in contrast to the above experiment by KATZ *et al.* the inhibitory transmitters liberated by impulses in fibres converging on to a motoneurone act at different receptor

sites on the motoneuronal membrane, namely on the subsynaptic areas under the respective synaptic knobs.

It may therefore be concluded that decisive evidence has been provided by the experimental tests of the four predictions derived from the hypothesis of an interneurone on the I a inhibitory pathway. No explanation of these results has been possible in terms of the alternative hypothesis of a monosynaptic inhibitory pathway.

Summary.

1. The establishment of the monosynaptic EPSP and the direct IPSP in relation to the I a volley has been compared with intracellular recording from motoneurones. The EPSP's appear at threshold stimulation of the I a afferents whereas an appreciable fraction of the I a afferents must be activated to evoke minimal observable direct IPSP's.

2. In some motoneurones a maximal I a volley from the nerve to an antagonist muscle may fail to produce a direct IPSP, but do so after post-tetanic activation.

With a nerve divided into many branches a maximal group I volley in any of them may not produce a direct IPSP in the motoneurone to an antagonist muscle whereas a synchronous volley in all of them evokes a large IPSP.

Occasionally evidence has been found suggesting that occlusion may also occur in the inhibitory pathway.

The findings are interpreted as being in strong support of the hypothesis postulating an interneurone in the direct inhibitory pathway.

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Paper Electrophoretic Studies on Serum Protein Changes in Blast Injury in Rabbits.

By

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In recent years electrophoretic separation methods have become an important tool in the study of changes in the pattern of blood serum or plasma proteins. Although only very few pathologic conditions are accompanied by specific changes in the electrophoretic pattern, the relation between the plasma protein components may be altered in a variety of diseases and posttraumatic conditions. Thus, it is now well known that there is during the acute phase of various infections and traumata a decrease of the albumin and an increase of the α_2-globulin and fibrinogen fractions (see *e. g.* OLHAGEN 1947, 1950). After severe trauma with extensive destruction of tissue, as for instance in burns the increase of the α_2-globulin fraction may be substantial.

CHANUTIN and co-workers have made a number of investigations on the changes in the concentrations of the different serum proteins of rats, dogs and goats after trauma. Thus, CHANUTIN and GJESSING (1946) report that in dogs exposure to hot water, freezing with dry carbon dioxide, subcutaneous injection of turpentine or bone fracturing cause a decrease of the albumin and an increase of the α-globulin fraction and a change of the appearance of the

γ-globulin pattern. The decrease of the albumin content was the earliest change of the protein pattern of serum that could be observed after the injury. Similar changes were observed after skin exposure to sulfur and nitrogen mustards (GJESSING and CHANUTIN 1946). In addition, the gas exposure gave rise to three α-globulin rich fractions which were not present in detectable amounts in the normal nonexposed dogs (GJESSING, LUDEWIG and CHANUTIN 1947).

In rats, GJESSING and CHANUTIN (1947) found an increase of the concentrations of α- and β-globulins and a decrease of the albumin and γ-globulin fractions during the first week following thermal injury. In goats, on the other hand, severe injury (burns, mustard gas exposure or turpentine injection) caused only relatively small changes of the serum protein pattern (GJESSING, LUDEWIG and CHANUTIN 1948).

In tourniquet shock in mice, MOORE (1953) found that the serum albumin rapidly decreased after the release of the tourniquet due to a leakage into the tissues at the site of injury. The α- and β-globulin fractions increased, the changes of the γ-globulin being inconsistent. The increase of the α- and β-globulins was interpreted as being due to an ultrafiltration of the serum at the site of injury.

CLEMEDSON (1949) observed in rabbits exposed to high explosive shock waves a lowering of the total plasma protein in severely as well as in slightly injured animals. As there was a similar decrease also in the nonexposed control animals the reduction of the plasma protein content was attributed chiefly to the blood letting. The relations between the various protein fractions were not studied. From the early works of CIJEK, ZUCKER and TOMPKINS (1930) and CIJEK and ZUCKER (1930) in dogs and the recent study by NIKLAS and HEMPEL (1954) in rats it can be seen that even an extreme blood loss is accompanied by only rather slight transient serum protein changes, chiefly a relative increase of the globulin fractions.

The present investigation was undertaken with the aim of studying the serum protein pattern in blast injury.

Methods.

About 60 rabbits weighing between 1.9 and 3.3 kg were used. They were anesthetized with urethane administered intravenously in the marginal ear vein in a dose of 1.4 to 1.5 g per kg body weight.

Shock wave exposure. The experimental animals were exposed to a high explosive shock wave in the detonation chamber described earlier by CLEMEDSON (1949) and CLEMEDSON and CRIBORN (1955). The high explosive charge used was an electric detonating cap and 2 g of a plastic explosive containing pentaerythrit tetranitrate (PETN). The electric blasting cap containing ca. 0.2 g of lead azide and lead trinitroresorcinate and 0.7 g of trinitrotoluene (TNT) has a blasting power which can be considered equivalent to one g of PETN. The maximum reflection overpressure obtained by the detonating cap plus 2 g of PETN, when measured by means of a mechanical membrane gauge (see CLEMEDSON 1949), is about 9 at.

Blood sampling. Blood for paper electrophoretic separation of the serum proteins was obtained by heart puncture which was the method found to be most suitable in order to prevent hemolysis. In some of the samples there occurred nevertheless a slight hemolysis. About 3 ml of blood were taken each time and allowed to coagulate at room temperature during one hour. The clot was then whipped with a glass rod, and the serum was separated by centrifugation. The serum obtained was stored in a refrigerator at +4° C until analysis.

Paper electrophoresis. An apparatus for paper electrophoresis was constructed. Electrophoresis was carried out at 80—100 volts (2—2.5 volts/cm) on Whatman filter paper no. 1. The buffer solution used consisted of diethyl barbituric acid (2.76 g/l) and the sodium salt of diethyl barbituric acid (15.45 g/l) in water. The pH of the buffer solution was 8.6, and the ionic strength 0.075. In each electrophoresis 0.02 ml of serum were used. The filter paper was immersed in the buffer solution and excess solution was wipped off. The moisted paper was allowed to gain equilibrium in the electrophoresis chamber for one hour before the serum was added. Each electrophoresis was run for 41 hours.

Dyeing and scanning. After the electrophoresis the papers were dried and stained with bromophenol blue according to a method described by JENCKS (1955). Scanning was carried out with an Elphor photometer after that the papers had been rendered transparent through immersion in α-bromonaphtalene. The reproducibility of the staining procedure was controlled by staining known amounts of albumin.

Statistical analysis. The material was statistically treated by means of analysis of variance.

Results.

In order not to render the animals anemic through too many blood lettings the experimental animals were divided into two groups, one in which blood samples were collected 6 hours and 1, 2 and 4 days after the detonation (Group I) and one group in which the first blood sample after the exposure was taken on the 5th day after the detonation and thereafter every second day until the

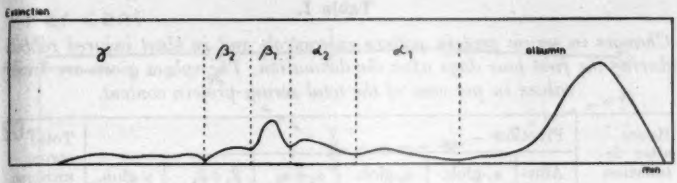

Fig. 1. Serum protein pattern of a control rabbit.

10th day (Group II). Two groups of control animals were treated in the same way as the experimental animals except that they were not exposed to the detonation.

Out of the total experimental animals, about 20 died of their blast injuries before the end of the observation period. All these animals have been excluded in the following analysis. The results to be described here are based on the observations of 16 animals in group I and 15 in group II.

Control animals.

In the normal rabbit it has been possible to separate six serum protein fractions. A paper electrophoretogram from a normal rabbit is shown in Fig. 1. In some cases, however, the fractions a_1 and a_2 and β_1 and β_2, respectively, could not be separated from each other. The mean percental composition of the serum protein of rabbits found in this study is in good agreement with those reported by other investigators (see *e. g.* STÜRMER 1951, SCHEIFFARTH and BERG 1952, PETER and HANSER 1953, CHIARIONI and NARDI 1956, HUDGINS, CUMMINGS and PATNODE 1956).

The control material comprising 13 animals was used chiefly to test if the repeated blood sampling would cause any changes in the normal serum protein pattern. No significant changes were found in the control animals belonging to Group II. In the group of animals, the serum protein patterns of which were studied during the three days immediately after the detonation (Group I), only 3 control animals were used. These animals showed some changes in their serum protein pattern that can be supposed to be due to the close sequence of blood lettings. The conclusions that will be drawn from the present investigation will, therefore, be based mainly on the findings in the animals belonging to Group II.

Table I.

Changes in serum protein pattern in controls and in blast injured rabbits during the first four days after the detonation. The values given are mean values in per cent of the total serum protein content.

Hours after detonation	Fraction						Total serum nitrogen mg per ml
	Albumin	α_1-glob.	α_2-glob.	$\alpha_1+\alpha_2$	$\beta_1+\beta_2$	γ-glob.	
0 (initial value)	51±2	9±0.6	9±0.7	18±1	17±1.3	15±1	10±0.5
6	55±2	8±0.6	7±0.7	15±1	16±1.3	13±1	9±0.5
24	53±2	9±0.6	9±0.7	20±1	16±1.3	10±1	9±0.5
48	46±2	12±0.6	10±0.7	24±1	20±1.3	10±1	10±0.5
96	44±2	14±0.6	—	22±1	20±1.3	12±1	10±0.5

Serum protein changes in blast injured animals.

Figures 2—6 show the changes in the serum proteins in the blast injured animals at various times up to 10 days after the detonation. The curves are based on values obtained in Group II animals only. The results from Group I are tabulated in Table I. The values given are the mean values in per cent of the total protein for each fraction analysed. As there was sometimes an only incomplete separation of the fractions $\alpha_1 + \alpha_2$ and $\beta_1 + \beta_2$, these fractions have also been calculated together.

Due to the small number of animals used and fairly large individual variations within each group the material was subjected to an analysis of variance.

The following general trend in the serum protein pattern was found in the injured animals.

The *albumin* fraction showed a decrease which was evident already two days after the detonation (Table I and Fig. 2). In some cases a slight increase preceded the lowering of the albumin concentration. The lowest values were found on the fourth to seventh day, a tendency to a return to normal values being found at the end of the observation period.

The *α_1-globulin* fraction (Table I and Fig. 3) was increased on the second day after the exposure (in the Group I animals) and the values remained above normal for some days. In the animals belonging to Group II a slight increase was noticed after 4 to 5

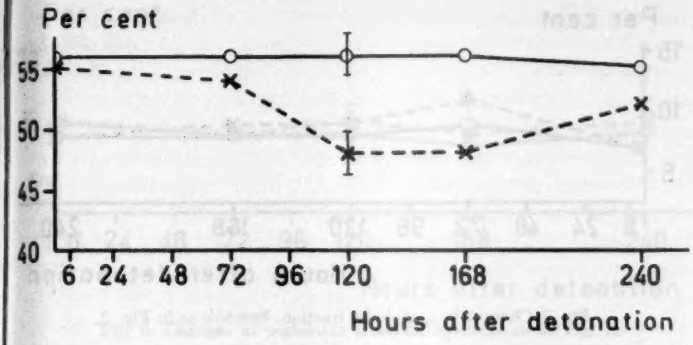

Fig. 2. Changes in albumin fraction after detonation. Values in per cent of total protein content. Symbols: —O— controls, --×-- blast injured animals.

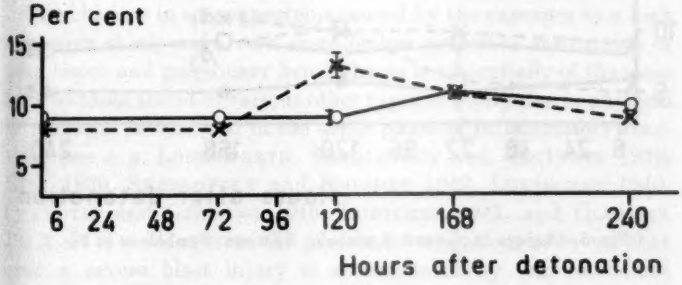

Fig. 3. Changes in α_1-globulin fraction. Symbols as in Fig. 2.

days. The *α_2-globulin* fraction (Table I and Fig. 4) showed a slight persisting increase.

The *β_1-globulin* fraction (Table I and Fig. 5) was not changed but in the *β_2-globulin* fraction (Table I and Fig. 5) a very strong increase was noted in some animals five days after the exposure. The individual variations were, however, very large as far as the β_1-globulin fraction is concerned and the increase of the β_2-fraction is not statistically significant ($P > 0.2$).

The *γ-globulin* fraction (Table I and Fig. 6), finally, was slihgtly reduced already on the day following that of the detonation, but already one or two days later the values were about the same as in the nonexposed controls.

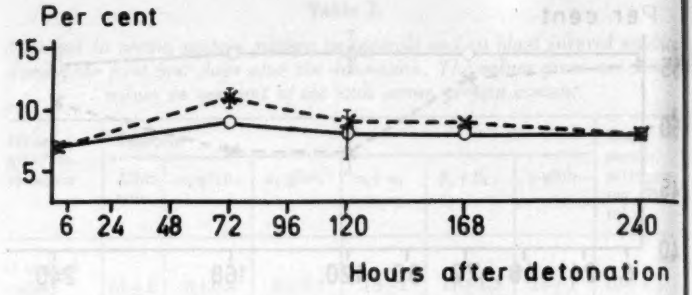

Fig. 4. Changes in α_2-globulin fraction. Symbols as in Fig. 2.

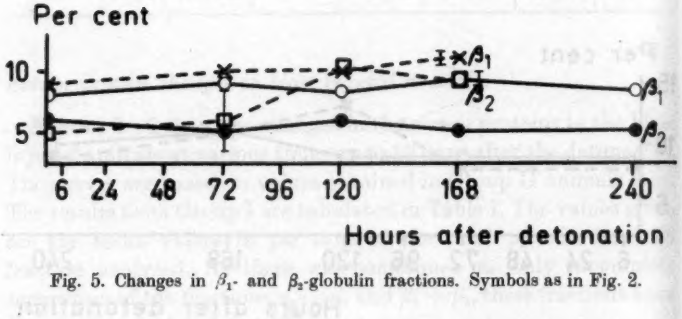

Fig. 5. Changes in β_1- and β_2-globulin fractions. Symbols as in Fig. 2.

The statistical treatment showed that the decrease in the albumin fraction, was probable ($0.025 > P > 0.01$). The early increase in α_1- and α_2-globulins was statistically significant ($P < 0.001$), the same being true for the reduction during the first days after the detonation of the γ-globulin fractions ($0.025 > P > 0.001$). In the Group II animals the increase in α_1- or α_2-globulins were not significant ($0.1 > P > 0.05$ for both fractions separately or calculated together). As far as the β-globulins are concerned the increase becomes statistically probable only if the fractions β_1 and β_2 are taken together ($0.025 > P > 0.01$).

No significant changes were found in the total serum nitrogen during the observation period either in the controls ($P > 0.5$) or in the experimental animals ($P > 0.3$).

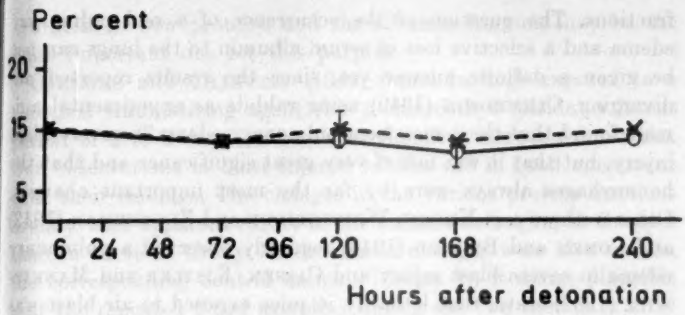

Fig. 6. Changes in γ-globulin fraction. Symbols as in Fig. 2.

Discussion.

The changes in serum proteins caused by the exposure to a high explosive shock wave with more or less extensive destruction of lung tissue and pulmonary hemorrhages are essentially of the same kind as those found in various other types of injury due to physical or chemical factors and in the acute phase of inflammatory reactions (see *e. g.* LONGSWORTH, SHEDLOVSKY and MACINNES 1939, BLIX 1939, SHEDLOVSKY and SCUDDER 1942, LUETSCHER 1940, CHANUTIN and GJESSING 1946, LUETCHER 1947, and OLHAGEN 1947). It is evident from the present investigation, however, that even a severe blast injury is accompanied by only moderate changes in the serum protein pattern.

The reduction of the albumin concentration is the earliest change seen in serum after various types of injury as far as the protein components are concerned (CHANUTIN and GJESSING 1946, LUETCHER 1947) and the degree of albumin reduction has been proposed as a measure of the severity of the injury. It has been supposed (CHANUTIN and GJESSING 1946) that the lowering of the albumin content could be due to a loss through damaged capillaries. In severe blast injury there is an extensive damage to the pulmonary capillary bed with inter- and intraalveolar hemorrhages which may give a two- to threefold increase of the weight of the lung. Hemorrhages *per se*, even very large ones, have been found in earlier investigations (CIJEK, ZUCKER and TOMPKINS 1930, CIJEK and ZUCKER 1930, NIKLAS and HEMPEL 1954), however, to cause no or only slight changes of the serum protein

fractions. The question of the occurrence of a real pulmonary edema and a selective loss of serum albumin to the lungs can not be given a definite answer yet, since the results reported are diverging. CLEMEDSON (1949) using rabbits as experimental animals found that there may be a pulmonary edema in severe blast injury, but that it was not of very great significance and that the hemorrhages always were by far the most important changes. Other authors *e. g.* KROHN, WHITTERIDGE and ZUCKERMAN (1942) and COHEN and BISKIND (1946) regularly observed a pulmonary edema in severe blast injury and CASSEN, KISTLER and MANKIEWICZ (1952) state, that lethality in mice exposed to air blast was always associated with extensive lung edema possibly of a central nervous origin. It seems unlikely though, that a leakage through damaged pulmonary capillaries is the only cause of the reduction of the serum albumin fraction in blast injury.

All albumin and at least 80 per cent of the globulins of plasma have been shown by MILLER et al. (1951) in experiments on perfused rat liver to be formed in the liver. If the turnover of the serum proteins is rather fast, an impairment of the formation of new blood proteins in the liver could be anticipated soon to lead to a reduction of these protein fractions in the circulating blood. In this connection some recent experiments on the turnover rate of serum proteins may be mentioned. VANSTONE et al. (1957) report the half-life of both serum albumin and globulin of cockerels to be about 6.5 days and in the pullet the half-lives were approximately 8.5 and 4.6 days, respectively. SUROVIKINA (1957) studying the rate of elimination of serum proteins from the blood circulation of rabbits found much shorter half-lives. The half-life of circulating albumin was determined to be 2.8 to 4.6 hours, that of α-globulin 2.3 to 4.6 hours and that of β-globulin 3.4 hours. SCHULTZE (1957) and SCHULTZE and MAURER (1957) give the values 4.1 and 6.8 days for rabbit β- and γ-globulins, respectively. The turnover rate, thus, is rather rapid and an impaired liver function due to circulatory disturbances (cf. CLEMEDSON 1949 and CLEMEDSON and HULTMAN 1958) during the acute phase of the blast injury could be anticipated to result in a lowering of the serum albumin content and a resulting increase in other fractions. A reduced synthesis due to inadequate food intake during that period or an increased destruction are other possible causes of the hypoalbuminemia. The body tissues are supposed to use serum proteins as a source of amino acids for the

synthesis of new proteins and the albumin fraction may be the most important one for this purpose.

GJESSING and CHANUTIN (1946) found in dogs exposed to a chemical traumatizing agent (bis β-chloroethyl sulfide) a latent period of 2 to 3 days before the α-globulins increased in serum. Our observations in blast injured rabbits are in good agreement with their findings. The changes in the various protein fractions usually were apparent earlier in the animals belonging to Group I than in those of Group II. As similar changes were also seen in the corresponding control animals (Group I), it seems plausible that the repeated blood sampling during the two first days after the exposure might at least partly have contributed to these changes.

The strikingly high β_2-globulin values found in some animals 3 to 5 days after the exposure may be due to nonremoved fibrinogen, as that protein localizes close to the β_2-fraction in the electrophoretogram. In severe trauma there may be a considerable increase in the fibrinogen content of plasma.

CHANUTIN and GJESSING (1946) have correlated the increase in the α-globulin content with formation of new protein fractions and GJESSING, LUDEWIG and CHANUTIN (1947) report the occurrence of three α-globulin rich serum fractions present in injured dogs but not in the control animals. Also in the β-globulin fraction such new protein may occur (GJESSING and CHANUTIN 1946). In the present material no abnormal protein fractions, possibly with one exception in one single aminal have been found, but due to the poor separation of the various γ-globulin fractions a qualitative change in the γ-globulin range is easily overlooked.

Summary.

1. The changes in the serum protein fractions in rabbits exposed to a high explosive air shock wave were studied by means of paper electrophoresis.

2. The albumin and γ-globulin concentrations were decreased, the albumin content during the whole observation period of 10 days after the exposure, the γ-globulin only during the first 4 days.

3. The α_1- and α_2-globulin fractions were slightly increased

from the second to the fifth day but had generally been normalized again at the end of the observation period.

4. In some cases a strong although statistically not significant increase of the β-globulin fractions was noticed. This increase can partly have been due to a contribution from incompletely removed fibrinogen.

5. No changes were found in the total nitrogen content of serum.

The authors' thanks are due to Stig Ek, M. Techn. and Ola Herzberg, Eng., Research Institute of National Defence, Dept. 1, for their valuable help with the statistical treatment of the material.

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A Percutaneous Method for Catheterization of the Renal Vein in Dogs.

By

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The technique for the obtaining of venous blood from a kidney has previously been based on the following principles:

1. By exposure or explantation of the relevant kidney (CORCORAN, SMITH and PAGE 1941).

2. By catheterization of the renal vein through the jugular vein (KREIENBERG 1954—55 and others).

The first alternative involves the risk that the circulatory conditions in the kidney will be disturbed and the walls of the catheterized renal vein thickened.

The other alternative necessitates exposure and ligation of the jugular vein. This technique precludes repeated examinations.

In recent years, chiefly as the result of SELDINGER's (1953) method and investigations, it has been possible to substitute percutaneous vascular catheterization for the majority of the methods based on exposure of a blood vessel. In order that a technique of this type may be carried out without too great difficulty the catheter must have a specially performed bend on its distal aspect,

adjusted to meet the anatomy of the particular vessel under examination, or, failing this, the distal part of the catheter must be capable of being arched by a guiding instrumentarium (RAPPAPORT 1952, PEARL, GRAY and FRIEDMAN 1950). In addition, there should be possibilities available for fluoroscopic control of the catheter.

A method fulfilling these requirements has been worked out by the present authors and tried out on dogs. It is carried out in the following manner.

A radio-opaque polythene catheter, size PE 160 and roughly 50 cm long, is used. Before the examination the distal end of the catheter is bent into an arch having a diameter of 2—3 cm. The arch should not be as large for catheterization of the cranial part of the kidney as when the caudal part is to be studied. The tip of the catheter is provided with two side holes having a calibre of approximately 1 mm and situated not more than 1—2 cm from the tip. These holes allow the contrast medium to flow freely through the catheter even if the distal bore becomes blocked. The latter situation may arise during catheterization, when the catheter tip presses against a vessel wall. The animals are examined under narcosis in order to avoid dislocation of the catheter through movement.

SELDINGER's method for catheter replacement is used for the introduction of the catheter into the venous system. The instruments originally recommended by SELDINGER (puncture needle and metal guide) have been used. The procedure is as follows: Before the puncturing, the femoral artery is palpated and fixed at a point slightly below the inguinal ligament. Not only does this reduce the risk for accidental puncturing of this vessel, but in addition the course of the femoral vein may be guessed at from the position of the artery. The puncture is made in a direction immediately medial to the artery, where the femoral vein is normally situated. After the vein has been punctured, a Seldinger guide, size PE 160, is introduced through the needle. The distal part of the guide will thus lie in the venous system. It should be inserted far enough to ensure that its distal extremity will lie in the inferior vena cava. The puncture needle is then removed by drawing it backwards over the guide, while the latter remains in position in the venous system. In place of the needle, the whole length of the catheter is "threaded" on to the guide. The catheter will thus follow the guide, so that when the guide and the catheter are pushed in, the

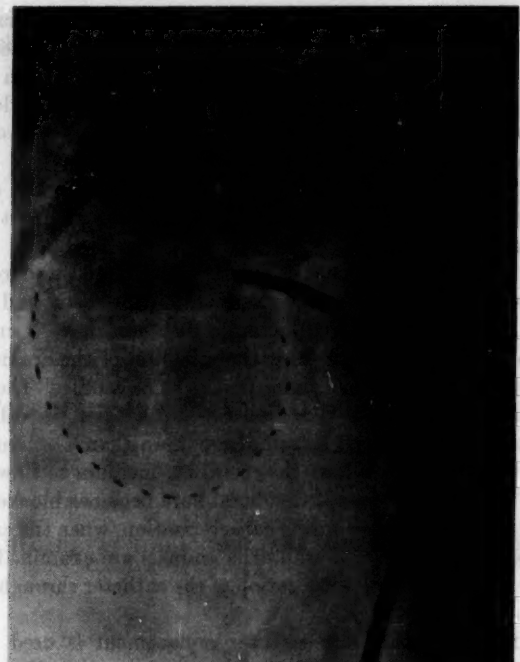

Fig. 1. Catheter placed in right renal vein.

catheter tip first penetrates the skin and then the wall of the femoral vein. The catheter's distal extremity will consequently lie within the vein. The catheter on the guide is then moved under fluoroscopic control until the tip lies in the inferior vena cava. The distal flexible extremity of the guide should be in the lead during this procedure, so that the distal bend of the catheter is held in a straightened-out position. If the catheter is in the lead, there is a risk that its distal bend will fasten in unimportant side branches. After it has been established fluoroscopically that the catheter tip is situated cranial to the transition between L. 1 and L. 2, *i. e.* the level at which the renal veins leave the inferior vena cava, the guide is withdrawn and the catheter is rinsed through.

The renal vein is then catheterized in the following manner.

The catheter tip is directed to one side or the other depending on which kidney is to be explored. The catheter is then drawn in a caudal direction while at the same time care is taken to see that the tip maintains its lateral direction. When the tip reaches the mouth of a vein, the catheter, because of the elasticity in its arch, finds its way into the vein. This may be observed immediately by posterio-anterior fluoroscopy, the bend of the catheter becoming suddenly accentuated and projecting outside of the area normally covered by the inferior vena cava (Fig. 1). In addition to this, the catheter pendulates in a cranio-caudal direction in time to the animal's respiration. The renal veins are as thick as a small finger. They are the only laterally directed branches of the inferior vena cava on a level with the upper part of the lumbar vertebral column of a calibre allowing catheterization with a radio-opaque polythene catheter of size PE 160. Thus, when it has been established by the afore-mentioned criteria that the catheter tip is lying in a tributary of the inferior vena cava, it may also be concluded that a *renal vein* has been catheterized. From this position, the catheter is pushed into the renal venous system until the tip lies far out towards the periphery and not in the main trunk of the renal vein. Immediately after the catheterization a continuous drip is arranged and kept in function during the remainder of the experiment. Blood tests or blood samples can then be taken. In order to check up on the catheter's position in relation to the renal venous system, retrograde phlebography is carried out after the samples have been taken. This is done by injecting 10 ml of 30 per cent Umbradil through the catheter by hand and after the injection exposing a film in frontal projection over the area around the catheter tip.

Discussion.

The method described here has been used on 15 dogs on thirty-eight occasions for the purpose of determining the extraction values for PAH clearance. No complications arose. Analysis of the blood samples indicated that the renal blood had not been intermingled with blood from other vessels. The results of these analyses are to be reported in a separate publication.

It is not difficult to carry out the examination and obtain blood samples. The actual catheterization of the kidney vein can usually be carried out without delay, *i. e.* within about one minute. In the

present series of examinations, a continuous drip through the catheter was arranged because of the risk of coagulation.

This catheterization method can be applied to both kidneys. In the present series, however, only the right kidney was studied. On the left side, the spermatic vein — or the ovarian vein — enters the renal vein at a point so far in, that this side is not suitable for the method, the risk that blood from other organs will be intermingled with the samples being considerable. This disadvantage probably also applies to other methods for procuring samples of renal blood which are based on catheterization of a renal vein. On the other hand, the method reported here, contrary to those previously described, is percutaneous and does not necessitate ligation of the introducing vein following the examination. Our method can therefore be used repeatedly.

Summary.

Catheterization of a renal vein in dogs is described. The method is based on SELDINGER's method for catheter replacement in intravascular catheterization, and the use of a premodelled radio-opaque polythene catheter.

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Spontan-rhythmische und unregelmässige Schwankungen der Leberdurchblutung des Menschen.

Von

KLAUS GRAF, WILHELM GRAF und SUNE ROSELL.

Eingegangen am 14. März 1958.

Versuche am narkotisierten Tier mit blutiger Messmethodik zeigen in verschiedenen Bezirken der Zirkulation unter Ruhebedingungen gewöhnlich ein recht gleichmässiges Stromzeitvolumen, welches mitunter nur geringe, meist druckpassive Änderungen im Traube-Hering-Mayer (THM)-Rhythmus (Periodendauer 7—30 sec, MATTHES 1951, WAGNER 1954, beim Menschen Periodendauer bevorzugt 8—15 sec, GOLENHOFEN und HILDEBRANDT 1957 d) aufweist. Fällt dagegen die Störung der physiologischen Verhältnisse durch Narkose und operative Schädigung fort, dann ist — auch unter Ruhebedingungen — nur selten eine Konstanz des Stromzeitvolumens zu finden. So wurden bei fortlaufender Haut- und Skeletmuskeldurchblutungsregistrierung am wachen Menschen häufig spontane (bisher in ihrer Ursache also unbekannte) Schwankungen bis zu 100% und mehr der Ruhedurchblutung mit einer Periodendauer von durchschnittlich 1 min gefunden, welche in Haut und Skeletmuskulatur zwar synchron, doch gegensinnig verliefen (SCHOOP und MARX 1956, GOLENHOFEN und HILDEBRANDT 1957 a). Auffälligerweise beteiligt sich der arterielle Blutdruck an solchen Schwankungen nicht (GOLENHOFEN und

HILDEBRANDT 1957 c). Die zuletzt zitierten Autoren haben nun die Möglichkeit diskutiert, dass Rückwirkungen dieser Spontanschwankungen auf den Gesamtkreislauf durch die Gegenläufigkeit von Haut- und Muskeldurchblutung kompensiert werden könnten. Sie vermuten aber, dass diese Kopplung der beiden grössten peripheren Kreislaufgebiete nicht der einzige Mechanismus für die Konstanthaltung des arteriellen Blutdrucks während des Ablaufs der spontanen Durchblutungsschwankungen sei, da auch bei völligem Fehlen der 1-Minutenrhythmen in der Haut diese im Muskel gut ausgeprägt sein können.

Ein wegen der Grösse seines Stromzeitvolumens sehr geeignetes Kompensationsgebiet ist die Intestinal- und Leberstrombahn. Diese beansprucht hier besonderes Interesse, weil orientierende Untersuchungen am wachen Menschen auch im Ablauf der Leberdurchblutung spontane Schwankungen gezeigt haben, die in Form und Periodendauer den Spontanschwankungen in Haut und Skeletmuskulatur ähnlich sind (BOCK, GRAF und HENSEL 1957).

Für die weitere Diskussion über eine mögliche Verbindung dieser Stromzeitvolumenschwankungen war eine bessere Kenntnis der Charakteristik der spontan Durchblutungsschwankungen der Leber — wie Periodendauer, Amplitude, Häufigkeit des Auftretens, Beeinflussbarkeit und Auslösbarkeit — wünschenswert. Die im folgenden beschriebenen Untersuchungen an 31 wachen Menschen haben daher vornehmlich der Beobachtung dieser Änderungen der Leberdurchblutung gedient. (Über die Ergebnisse wurde bereits kurz berichtet, GRAF et al. 1957). Ein zeitlicher Zusammenhang im Ablauf von Durchblutungsschwankungen in Leber, Haut, Skeletmuskulatur u. a. m. kann hingegen nur durch synchrone Durchblutungsregistrierungen in diesen Kreislaufgebieten festgestellt werden. Derartige Messungen sind im Gange.

Methodik.

Die lokale Leberdurchblutung wurde mit einer modifizierten, flexiblen HENSEL'schen Wärmeleitsonde (WLS) fortlaufend registriert. Die für die Berechnung der Wärmeleitzahl ($\lambda = K \cdot I^2 \vartheta$) erforderliche Konstante K wurde für jede WLS (im Ganzen verwandten wir 4 WLS gleicher Bauart) bei 20° C in 10 % Gelatine bestimmt (Gelatina alba pulvis, Hersteller: E. Merck, Darmstadt. Auflösung der Gelatine in Aqua destillata). Jede WLS wurde hintereinander in 3 jeweils frisch zubereiten Gelatinelösungen kalibriert. Als endgültige Konstante K

diente der Mittelwert aller für jede WLS erhaltenen Einzelwerte. Unter Annahme einer Wärmeleitzahl für 10 % Gelatine von $12.1 \cdot 10^{-4}$ $cal \cdot cm^{-1} \cdot sec^{-1} \cdot °C^{-1}$ (Grayson 1952) lagen die errechneten Werte für K zwischen 0.010 und 0.021.

Die Registrierung der Thermospannungen der WLS erfolgte über ein Spiegelgalvanometer, welches einen Direktschreiber zur kontinuierlichen Aufzeichnung der Galvanometerausschläge (Nachlaufschreiber, Hersteller: Fa. Dr. B. Lange, Berlin) steuerte. Die Einstellzeit des gesamten Registriersystems für 95 % des Endwertes betrug 8 sec, die Registriergeschwindigkeit 22 mm/min. Die 1 mm dicke, sterilisierte WLS wurde beim liegenden Patienten bei einer Nadelbiopsie der Leber nach Entnahme der Gewebsprobe durch die Punktionskanüle in die rechte Leberhälfte eingeführt (Graf, Porjé und Allgoth 1954). Die Punktionsstelle war zuvor mit 1 % Xylocain ® ausreichend anästhesiert worden. Vor dem Zurückziehen der Punktionskanüle über die in der Leber verbleibende WLS wurde versucht, die starre Spitz der WLS nach Möglichkeit noch einige Millimeter über die Kanülenspitze hinaus in noch nicht durch die Punktion geschädigtes Gewebe vorzuschieben. Gemessen von der Eintrittsstelle in die Haut befand sich die WLS 10.0—23.5 cm (Mittelwert: 14.5 cm) tief im Körper. Röntgenaufnahmen in 2 Ebenen, die in einigen Fällen angefertigt wurden, zeigten, dass bei diesen Patienten die Spitze der WLS innerhalb des Leberparenchyms lag. Da die Durchblutungsmessung nur an der Spitze der WLS stattfindet, kann angenommen werden, dass nur die Leberdurchblutung von der Messung erfasst wurde. Ein Anhaltspunkt für die Lage der WLS ergab sich auch aus der Höhe der Wärmeleitzahl, die in der Leber relativ gross ist. (Die ausführliche Beschreibung der Wärmeleitzahlmessung in der Leber, den Aufbau der flexiblen WLS und das Vorgehen bei der Messung am Menschen siehe bei Graf, Golenhofen und Hensel (1957) und Bock, Graf und Hensel (1957).

Die fortlaufende Registrierung begann unmittelbar nach dem Einführen der WLS und dauerte 60 bis 360 (Mittelwert: 220) min. Bei den meisten Versuchen wurde nach einiger Zeit durch zentimeterweises Herausziehen der WLS die Sondenlage ein- bis mehreremale verändert. Auf diese Weise ergaben sich bei insgesamt 31 Versuchen 70 Sondenlagen.

Versuchsbedingungen.

Die Untersuchungen erfolgten an 31 Patienten im Alter von 24—72 (Mittelwert: 49) Jahren, die sich wegen eines vermuteten oder bereits gesicherten Leberleidens in klinischer Behandlung befanden. Im Verlaufe des Kliniksaufenthaltes zeigte die routinemässige klinische Leberdiagnostik in Verbindung mit dem Befund der Leberbiopsie:

1. bei 16 Patienten keinen Leberschaden,
2. bei 9 Patienten eine Fettleber verschieden starken Grades,
3. bei 3 Patienten eine Laennec'sche Cirrhose,
4. bei 2 Patienten ein postikterisches Bild und

5. bei 1 Patient eine terminolaterale Anastomose zwischen V. portae und V. cava caud., die 6 Monate zuvor wegen stärkerer portaler Hypertension bei fortgeschrittener Laennec'scher Cirrhose operativ angelegt worden war.

Die Untersuchungen fanden unter folgenden *Ruhebedingungen* statt: Die Patienten lagen — auf dem Rücken oder auf der linken Seite — in einem weichen Untersuchungsbett. Sie waren ausreichend zugedeckt. Alles, was zu Unbehagen führen konnte, wurde sorgfältig vermieden. Die Zimmertemperatur betrug 20—21° C. Die Punktionsstelle mit dem Eintritt der WLS wurde mit 1 % Xylocain ® so tief anästhesiert, dass auch tiefe Atemexcursionen keine Schmerzen mehr verursachten. Den Patienten wurde bedeutet, ruhig zu liegen und sich nicht zu unterhalten. Essen und Trinken war während der Registrierung nicht gestattet, dagegen konnten die Patienten nach Belieben lesen oder schlafen. Viele Patienten schliefen auch wirklich im Laufe der Untersuchung, teils mehrfach, unter vertiefter Atmung und auch Schnarchen fest ein (s. Abb. 4, Kurve 1). Seitens der Experimentatoren wurden Unterhaltungen und laute Manipulationen wie alles, was den Ruhezustand der Patienten stören konnte, nach Möglichkeit vermieden.

Bei allen Versuchen wurden gegen Ende der Registrierung intravenöse oder subcutane Injektionen von kreislaufwirksamen Substanzen vorgenommen. Um auch deren Wirkung von einer Ruhelage ausgehen zu lassen, erfolgten fast sämtliche i. v. Injektionen und Infusionen durch einen zu Versuchsbeginn gelegten Katheter in die V. cubitalis. Das freie Ende dieses langen Katheters befand sich hinter dem Kopfende des Untersuchungsbettes, von wo aus Injektionen und Infusionen ohne Kenntnis des Patienten beginnen konnten. Psychisch bedingte Einflüsse auf die Zirkulation, die z. B. beim Muskel zu dem typischen Bild der Adrenalinwirkung führen können (GOLENHOFEN und HILDEBRANDT 1957 b), sollten auf diese Weise vermieden werden.

Insgesamt wurden 119 mal Wirkstoffe injiziert, und zwar: Histamin, Adrenalin, Noradrenalin, ATP (Adenosin-tri-Phosphorsäure), Vasopressin (Pitressin) und Thiopentone (Thiopenton-Natrium) (Näheres s. Tab. I).

Ergebnisse.

I. Spontan-rhythmische Schwankungen der Leberdurchblutung.

a. Auffälligster Befund beim fortlaufenden Registrieren der lokalen Leberdurchblutung des Menschen ist das Vorhandensein von Schwankungen, wie sie in Abb. 1 wiedergegeben sind. Jede dieser 8 Kurven ist ein Beispiel aus einem anderen Versuch. Diese Schwankungen erfolgten ohne erkennbare Ursache als ein „in stetigem Fluss befindliches, gegliedertes Geschehen" und können nach dieser Definition von WACHHOLDER (1955) daher

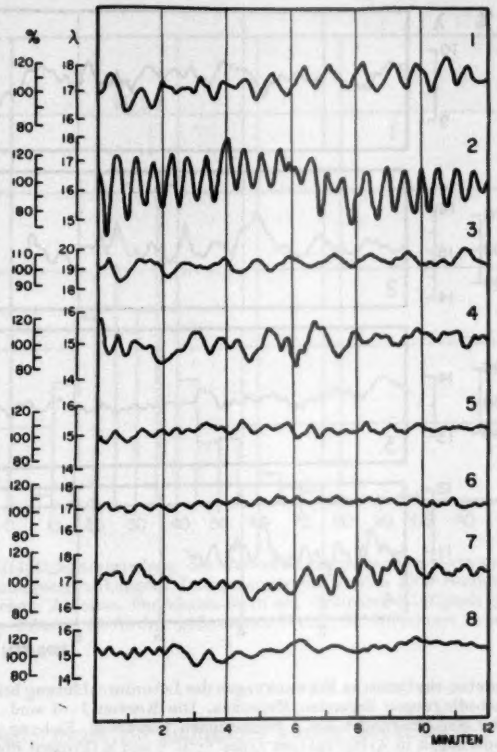

Abb. 1: Spontan-rhythmische Schwankungen der Leberdurchblutung beim wachen, unter Ruhebedingungen liegenden Menschen. Die Kurven 1—8 sind Abschnitte fortlaufender Registrierungen aus 8 verschiedenen Versuchen. Eichung der Durchblutungsänderungen in λ (10^{-4} cal · cm^{-1} · sec^{-1} · $°C^{-1}$) und % (Prozent der mittleren Ruhedurchblutung).

als *spontan-rhythmische Leberdurchblutungsschwankungen* bezeichnet werden. Nicht zu beobachten waren sie nur bei einem Patienten, bei dem die portale Leberdurchblutung 6 Monate vorher wegen Lebercirrhose durch einen termino-lateralen porto-cavalen Shunt unterbrochen worden war. Hier konnten sie auch nach viermaliger Änderung der Sondenlage durch zentimeterweises Herausziehen der WLS nicht festgestellt werden. Im Einzelversuch waren die Schwankungen unterschiedlich lange vorhanden, nur in 2 Versuchen dauerten sie fast während der gesamten Regi-

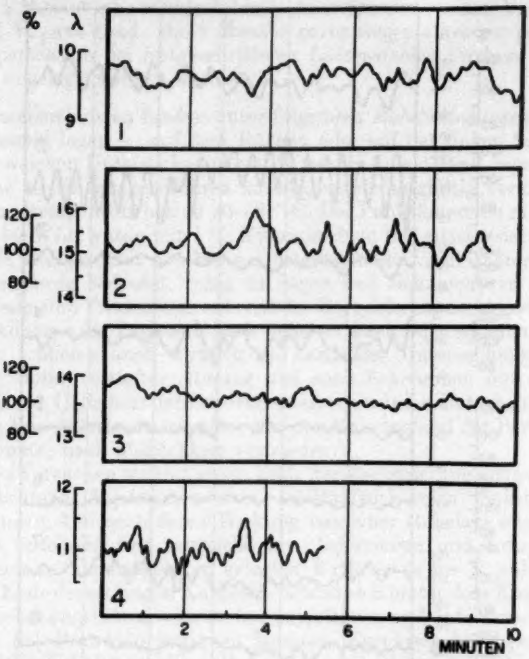

Abb. 2: Spontan-rhythmische Schwankungen der Leberdurchblutung beim wachen, unter Ruhebedingungen liegenden Menschen. Die Kurven 1—4 sind Abschnitte fortlaufender Registrierungen aus 4 verschiedenen Versuchen. Eichung der Durchblutungsänderungen in λ (10^{-4} cal · cm^{-1} · sec^{-1} · $°C^{-1}$) und % (Prozent der mittleren Ruhedurchblutung). An den Kurven 1 und 4 wurde eine Prozenteichung nicht angebracht, da diese Versuche an verfetteten Lebern erfolgten.

strierung von 220 und 250 min an. In anderen Versuchen aber waren sie nur wenige Minuten zu beobachten. Zusammengerechnet bestanden sie im Durchschnitt aller Versuche mit einer Amplitude über 5 % der mittleren Ruhedurchblutung während etwa 30% der Gesamtversuchsdauer und mit einer Amplitude über 2% der mittleren Ruhedurchblutung während etwa 60% der Gesamtversuchsdauer. In einigen Fällen waren sie unmittelbar nach dem Einführen der WLS zu messen, in anderen Fällen war die Durchblutung zu Beginn der Registrierung gleichmässig und die rhythmischen Schwankungen entstanden erst im Laufe von 1 Stunde nach Einführung der WLS. Waren sie auch dann noch nicht vorhanden,

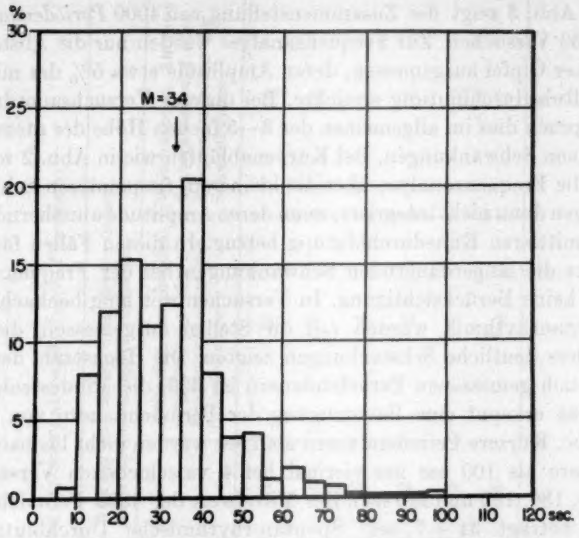

Abb. 3: Häufigkeitsverteilung der Periodendauern spontan-rhythmischer Leberdurchblutungsschwankungen. Zusammengestellt wurden 4000 Periodendauern aus 30 Versuchen. Abscisse: Periodendauer in sec. Ordinate: Häufigkeit der Periodendauern in Prozent sämtlicher gemessenen Werte. M: Mittelwert in sec.

so genügte meist ein einmaliges kurzes Zurückziehen der WLS in eine andere Lage, um sie schliesslich doch noch beobachten zu können. Häufig wurden sie von unterschiedlich langen Perioden gleichmässiger Leberdurchblutung unterbrochen. Von den atemsynchronen, sehr kleinen Schwankungen waren sie durch Periodendauer und Amplitude einwandfrei abzutrennen (s. dazu Bock, Graf und Hensel 1957), zumal die Trägheit der Registrierung so gewählt war, dass über die atemsynchronen Schwankungen weitgehend integriert wurde. Viele Kurven, wie Nr. 1, 3, 5 und 6 in Abb. 1 lassen eine weitgehend gleichförmigen Ablauf der Schwankungen erkennen. Andere zeigten aber auch plötzliche, in ihrer Ursache unbekannte Änderungen der Amplitude (Kurven 2 und 8 in Abb. 1) oder der Periodendauer (Kurve 8 in Abb. 1). In einigen Fällen waren kleinere, schnellere Schwankungen zu beobachten, welche langsameren Schwankungen mit grösserer Amplitude superponiert waren, besonders deutlich z. B. in Abb. 2.

b. Abb. 3 zeigt die Zusammenstellung von 4000 *Periodendauern* aus 30 Versuchen. Zur Frequenzanalyse wurden nur die Abstände solcher Gipfel ausgemessen, deren Amplitude etwa 5% der mittleren Ruhedurchblutung erreichte. Bei unserer Versuchsanordnung entsprach dies im allgemeinen der 3—5 fachen Höhe der atemsynchronen Schwankungen. Bei Kurvenabläufen wie in Abb. 2 wurde für die Frequenzanalyse über die kleineren, frequenteren Schwankungen dann nicht integriert, wenn deren Amplitude annähernd 5% der mittleren Ruhedurchblutung betrug. In diesen Fällen fanden daher die längerdauernden Schwankungen bei der Frequenzanalyse keine Berücksichtigung. In Versuchen mit lang beobachteter Spontanrhythmik wurden nur die Stellen ausgemessen, die besonders deutliche Schwankungen zeigten. Die Höchstzahl der pro Versuch gemessenen Periodendauern ist 339, die Mindestzahl 18.

Man erkennt eine Bevorzugung der Periodendauern von 10—45 sec. Kürzere Periodendauern als 7 sec wurden nicht beobachtet, längere als 100 sec nur viermal bei 4 verschiedenen Versuchen (160, 180, 195 und 220 sec). Der Mittelwert der 4000 Periodendauern beträgt 34 ± 7 sec. Spontan-rhythmische Durchblutungsschwankungen mit längerer Periodendauer als 220 sec, welche, etwa mit 4—8 minütiger Dauer, an der Hundeleber gemessen worden sind (GRINDLAY, HERRICK und MANN 1941, GRAF, GOLENHOFEN und HENSEL 1957), haben wir bei diesen Untersuchungen nicht gesehen.

c. Die *Amplitude* der spontan-rhythmischen Schwankungen betrug im Mittel der Schwankungen, die grösser als 5 % der mittleren Ruhedurchblutung waren, etwa 15 % der mittleren Ruhedurchblutung. Die maximalen Amplituden erreichten 70 %. Es wurden nicht sämtliche Amplituden ausgemessen, sondern nur diejenigen, deren Grösse auch für die umliegenden Schwankungen repräsentativ erschien. Die in Fettlebern gemessenen Werte wurden für diese Berechnung nicht verwandt.

d. Eine *Beeinflussung* der spontan-rhythmischen Durchblutungsschwankungen durch Pharmaka wie Histamin, Adrenalin, Nor-Adrenalin, ATP, Vasopressin und Thiopentone konnte in einer Reihe von Fällen beobachtet werden (Tab. 1). In Erweiterung der Befunde von BOCK, GRAF und HENSEL (1957) sahen wir einige Male auch spontan-rhythmische Schwankungen auftreten, wenn solche vorher nicht zu beobachten gewesen waren, oder in anderen Fällen eine gänzliche Unterdrückung vorhandener Wellen. Beispiele für den ersten Fall sind in Abb. 4 enthalten. 6 min nach Einsetzen

Tabelle I.

Beeinflussung spontan-rhythmischer Leberdurchblutungsschwankungen durch Pharmaka.

Stoff	Dosis pro kg. Körpergewicht	Gesamtzahl der Injektionen	Zahl der Patienten	Wirkung auf die spontan-rhythmischen Leberdurchblutungsschwankungen		
				Keine	Auftreten, Amplitudenvergrösserung oder Frequenzabnahme der Schwankungen	Verschwinden, Amplitudenverkleinerung oder Frequenzzunahme der Schwankungen
Histamin	10—16 μg i. v. oder 0.1—1.0 μg/min i. v.	31	14	11	16	4
Nor-Adrenalin ...	0.06—0.75 μg/i. v. oder 0.1—0.7 μg/min i. v.	26	11	15	5	6
Adrenalin	0.08—0.25 μg i. v. oder 0.04—0.40 μg/min i. v	26	11	16	4	6
ATP	10—3000 μg i. v. oder 20—240 μg/min i. v.	28	10	22	1	5
Vasopressin	0.3 IE/min i. v. oder 20 IE s. c.	4	4	3	1	—
Thiopentone	3—6 mg i. v.	4	4	1	2	1

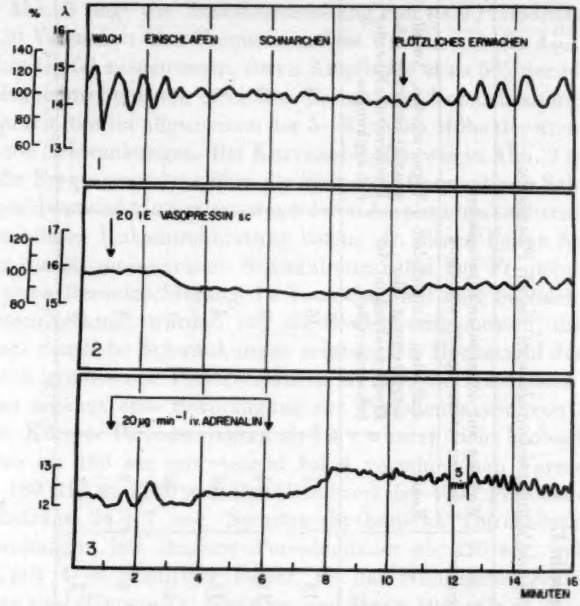

Abb. 4: Abschnitte fortlaufender Registrierungen der Leberdurchblutung des Menschen aus 3 verschiedenen Versuchen. Man beachte das Zurückgehen der Spontanschwankungen während des Einschlafens (Kurve 1) und das Auftreten von Spontanschwankungen nach dem Wirkungseintritt von 20 IE s. c. Vasopressin (Kurve 2) und 20 µg/min. i. v. Adrenalin (Kurve 3). Eichung der Durchblutungsänderungen in λ (10^{-4} cal · cm^{-1} · sec^{-1} · °C^{-1}) und % (Prozent der mittleren Ruhedurchblutung).

der Vasopressinwirkung mit Abnahme der Leberdurchblutung (Kurve 2) oder 10 min nach i. v. Adrenalininfusion mit nachfolgender Zunahme der Leberdurchblutung (Kurve 3) traten deutliche spontan-rhythmische Durchblutungsschwankungen auf, die in dieser Charakteristik bis dahin nicht bestanden hatten. Viel häufiger wurden unter der Einwirkung der Pharmaka aber lediglich Periodendauer oder Amplitude der Schwankungen verändert, wobei es gewöhnlich zu einer Abnahme der Periodendauer und einer Verkleinerung der Amplitude kam. Auch ohne Änderung der mittleren Leberdurchblutung konnten die Schwankungen beeinflusst werden, so vor allem nach i. v. Injektionen und Infusionen von Adrenalin (0.08—0.25 µg/kg oder 0.04—0.40 µg/

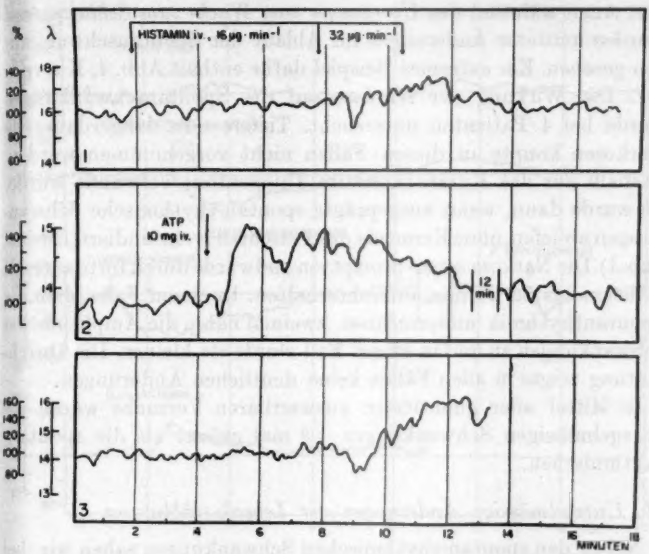

Abb. 5: Abschnitte fortlaufender Registrierungen der Leberdurchblutung des Menschen aus 3 verschiedenen Versuchen. Die Charakteristik der jeweiligen Spontanschwankungen bleibt auch auf einem höheren Durchblutungsniveau nach 16 und 32 µg/min Histamin i. v. (Kurve 1), nach 10 mg i. v. ATP (Kurve 2) oder nach Entstehung aus unbekannter Ursache (Kurve 3) praktisch unverändert. In der Wiedergabe der Kurve 2 wurde eine Strecke von 12 min Registrierdauer ausgelassen, die gleiche Spontanschwankungen wie in den dargestellten Kurvenabschnitten zeigte. Eichung der Durchblutungsänderungen in λ (10^{-4} cal · cm^{-2} · $sec.^{-1}$ · $°C^{-1}$) und % (Prozent der mittleren Ruhedurchblutung).

kg/min) und Nor-Adrenalin (0.06—0.75 µg/kg oder 0.—0.7 µg/kg/min), die ebenfalls gewöhnlich zu einer Abnahme der Periodendauern und Verkleinerung der Amplituden führten.

Tab. 1 zeigt, dass die verwandten Pharmaka in der Mehrzahl der Fälle jedoch keine deutliche Beeinflussung der Spontanrhythmik erkennen liessen. Dazu gibt Abb. 5 einige typische Beispiele, in denen die Schwankungen auch auf dem nach Histamin- und ATP-Injektionen veränderten Durchblutungsniveau in praktisch unveränderter Grösse und Periodendauer fortbestanden. Gleiches konnte auch dann beobachtet werden, wenn sich das Durchblutungsniveau ohne erkennbaren Grund veränderte (Abb. 5, Kurve 3).

e. Auch während des *Überganges vom Wach- zum Schlafzustand* wurden mitunter Änderungen im Ablauf der Spontanschwankungen gesehen. Ein extremes Beispiel dafür enthält Abb. 4, Kurve 1.

f. Die Wirkung der *Narkose* auf die Spontanschwankungen wurde bei 4 Patienten untersucht. Tiefere oder längerdauernde Narkosen konnte in diesen Fällen nicht vorgenommen werden, weshalb nur das Kurznarkotikum Thiopentone verwandt wurde. Es wurde dann, wenn ausgeprägte spontan-rhythmische Schwankungen abliefen, ohne Kenntnis des Patienten iv. infundiert (Dosis s. Tab. 1). Die Narkose setzte prompt ein und wurde durch fortdauernde Infusion bis zu 20 min aufrechterhalten. In einem Falle blieb die Spontanrhythmik unbeeinflusst, zweimal nahm die Amplitude der Schwankungen zu und in einem Fall wurde sie kleiner. Die Durchblutung zeigte in allen Fällen keine deutlichen Änderungen.

In Mittel aller quantitativ auswertbaren Versuche waren die unregelmässigen Schwankungen 1.8 mal grösser als die spontanrhythmischen.

II. Unregelmässige Änderungen der Leberdurchblutung.

Neben den spontan-rhythmischen Schwankungen sahen wir bei jedem Versuch *unregelmässige Änderungen der Leberdurchblutung.* Meist waren es rasche Abnahme der Durchblutung auf 90—70% der mittleren Ruhedurchblutung, die danach nur allmählich während 2—30 min oder noch längerer Zeit wieder zum Ausgangswert zurück- oder in einen anderen gleichbleibenden Durchblutungswert übergingen. Spontane Durchblutungsanstiege wie in Abb. 5, Kurve 3 wurden nur selten beobachtet. Diese unregelmässigen Änderungen traten meist bei nur geringfügigen Änderungen der Körperlage, bei Schmerzempfindungen und bei solchen Situationen auf, die zu gesteigerter Erwartungsspannung führten wie plötzliche laute Geräusche oder auffällige Manipulationen der Experimentatoren. Hier ist es berechtigt, sie als „reaktive" Durchblutungsänderungen zu definieren. In manchen Fällen (Abb. 5, Kurve 3) war ein auslösendes Ereignis für diese Durchblutungsänderungen aber nicht zu bemerken gewesen, was daran liegen kann, dass z. B. psychische Reaktionen der Patienten leicht der Beobachtung entgehen.

Die Grösse dieser unregelmässigen Durchblutungsänderungen betrug unter unseren Versuchsbedingungen maximal 75% der am Versuchsende gemessenen mittleren Ruhedurchblutung.

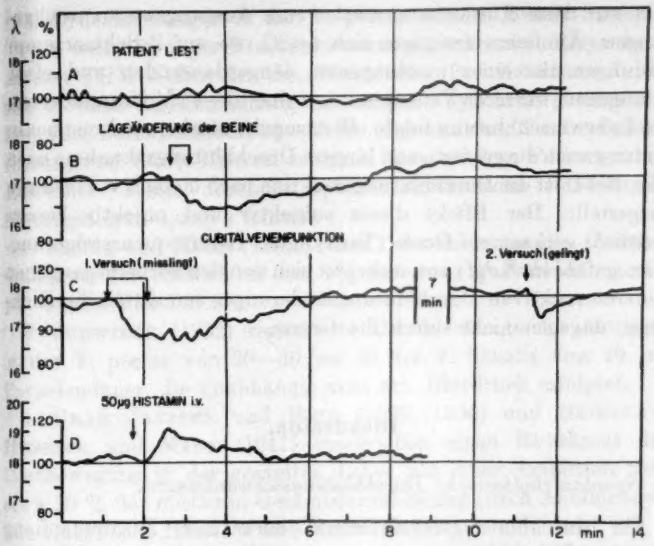

Abb. 6: Unregelmässige, reaktive Schwankungen der Leberdurchblutung des Menschen. Alle Kurven stammen aus demselben Versuch. Die Leberdurchblutungsänderungen nach 50 µg i. v. Histamin ist den Schwankungen nach Lageänderung der Beine und Schmerzempfindungen vergleichsweise gegenübergestellt. Eichung der Durchblutungsänderungen in λ (10^{-4} cal · cm^{-1} · sec^{-1} · $°C^{-1}$) und % (Prozent der mittleren Ruhedurchblutung).

Abb. 6 zeigt diese unregelmässigen, hier sicherlich reaktiven Schwankungen in einigen typischen Beispielen, die alle demselben Versuch entnommen sind. Bei A las der auf dem Untersuchungsbett liegende Patient eine Zeitung, ohne Zeichen körperlicher Unruhe oder psychischer Erregung. Die Leberdurchblutung wies währenddessen nur geringe, sehr langsame, unregelmässige, in dieser Form öfters zu beobachtende Schwankungen um einen gleichbleibenden Mittelwert auf. Andeutungen von spontanrhythmischen Schwankungen der Leberdurchblutung sind in der Kurve vorhanden. Bei B erfolgte im Liegen eine geringfügige Lageänderung beider Beine, bei der innerhalb von etwa 30 sec beide Kniee zur Entspannung etwas angezogen und dann gleich wieder ausgestreckt wurden. Von Beginn dieser Lageänderung ab erfolgte eine Abnahme der zuvor praktisch konstanten Leberdurchblutung auf 90% der Ausgangsdurchblutung. Erst nach 5

min war diese Abweichung wieder zum Ausgangswert zurückgegangen. Ähnliches ereignete sich bei C, wo auf 2 Schmerzempfindungen bei einer misslungenen, längerdauernden und einer gelungenen, kürzeren Venenpunktion eine 10—15 % Verminderung der Leberdurchblutung folgte. Hier zog die stärkere Schmerzempfindung auch die grössere und längere Durchblutungsabnahme nach sich. Bei D ist die Durchblutungsreaktion nach 50 μg i. v. Histamin dargestellt. Der Effekt dieser subjektiv und objektiv bereits deutlich wirksamen Dosis (Tachypnoe, Herzfrequenzsteigerung, Hitzegefühl im Kopf) unterscheidet sich von den vorhergegangenen anderen reaktiven Durchblutungsänderungen nur durch die Richtung, dagegen nicht durch die Grösse.

Diskussion.

I. Spontan-rhythmische Durchblutungsschwankungen.

Ein durchblutungsregulatorisch wirksamer Zusammenhang zwischen Durchblutungsänderungen in Leber, Haut und Skeletmuskulatur verlangt, dass die Änderungen in grösseren Teilen dieser Kreislaufgebiete synchron zueinander erfolgen. Aufsichtsmikroskopisch wurden aber bereits für die kleinste zirkulatorische Einheit der Leber, den Lobulus, Blutgehalts- und Durchblutungsschwankungen beobachtet, die in Minutenfristen in jedem einzelnen Lobulus und sogar in den einzelnen Gefässen, den Sinusoiden, asynchron zu den Schwankungen in den Nachbarsinusoiden und Nachbarlobuli ablaufen (Loeffler und Nordmann 1925, Wakim und Mann 1942 und 1953, Knisely, Bloch und Warner 1948, Seneviratne 1950, Irwin und Macdonald 1953, Burrage et al. 1953, Burrage und Irwin 1953, Peters 1956). Die Wärmeleitmessung erfasst aber mit Sicherheit die Durchblutungsänderungen in einer grösseren Zahl von Lobuli, weshalb die registrierten Schwankungen nicht auf lokale lobuläre Durchblutungsänderungen bezogen werden können. Befunde anderer Autoren zeigen zudem, dass auch in grösseren Teilen der Leber synchrone Durchblutungsschwankungen ablaufen können:

a. Neumayer (1957) beobachtete bei fortlaufender thermoelektrischer Durchblutungsregistrierung in der V. hepatica des Menschen spontan-rhythmische Schwankungen der Durchblutung von 10—15 % der mittleren Ruhedurchblutung.

b. Druckmessungen in der V. portae wacher Hunde liessen 2 Typen wellenförmiger Druckschwankungen erkennen, 1) synchron zu den THM-Wellen des art. Blutdrucks, Periodendauer: 7—14 sec, 2) ohne Zusammenhang mit anderen synchron gemessenen Drucken, Periodendauer: 30—55 sec (GERSMEYER, GASTEYER und GERSMEYER 1957). Hier sei erwähnt, dass bei dem Patienten mit Eck'scher Fistel deutliche spontan-rhythmische Leberdurchblutungsschwankungen fehlten.

c. SEMBA und KISHI (1952) sahen Schwankungen des Gesamtvolumens der Hundeleber um 1% mit einer Periodendauer von 15—38, im Mittel 28 sec.

d. SCHWIEGK (1932) registrierte Durchblutungsschwankungen in der V. portae von 20—30 sec in der V. lienalis von 40 sec Periodendauer, die unabhängig vom art. Blutdruck erfolgten.

e. GRAB JANSSEN und REIN (1929, 1930) und GRINDLAY, HERRICK und MANN (1941) beschrieben einen Rhythmus der Blutbewegung in der gesamten Leber mit einer Amplitude von etwa 20 % der mittleren Gesamtdurchblutung (nach Abbildungen von GRINDLAY, HERRICK und MANN 1941).

f. Mit der Bromsulphaleinemethode wurden im Ruhezustand Schwankungen der geschätzten Leberdurchblutung gemessen, die beim Menschen bis zu 20 %, beim Hund bis zu 30 % der mittleren Ruhedurchblutung betrugen (WERNER und HORVATH 1952, BOLLMAN et al. 1953, MCSMYTHE, GILMORE und HANDFORD 1954, BRADLEY 1956).

Auch in anderen abdominalen Organen wurden den spontan-rhythmischen Leberdurchblutungsschwankungen in Form und Periodendauer ähnliche Durchblutungs- und Volumenschwankungen beobachtet. DEMLING und GROMOTKA (1957) beschrieben spontan-rhythmische, häufig von kleineren und schnelleren Oberwellen überlagerte Durchblutungsschwankungen im Rectum und Sigma des wachen Menschen von 30—90, maximal 240 sec Dauer und einer Amplitude von 5—25, maximal 65 % der mittleren Ruhedurchblutung. PRILL (1958) registrierte ähnliche Durchblutungsschwankungen im menschlichen Myo- und Endometrium. SEMBA und KISHI (1951) beschrieben spontan-rhythmische Volumenschwankungen der Hundeniere mit einer mittleren Periodendauer von 21 sec.

Alle diese spontan-rhythmischen Durchblutungs- und Volumenschwankungen sind in der Form des Ablaufes und der Frequenz den von GOLENHOFEN und HILDEBRANDT (1957 a) beschriebenen

spontan-rhythmischen Durchblutungsschwankungen in Haut und Skeletmuskulatur sehr ähnlich. Die Schwankungen in Haut und Skeletmuskulatur sind zeitlich koordiniert. Es ist vorstellbar, dass auch die abdominellen spontan-rhythmischen Durchblutungsschwankungen an dieser zeitlichen Koordination teilhaben und mit den Schwankungen in Haut und Skeletmuskulatur synchron ablaufen. Der Effekt der eingangs vermuteten Durchblutungsregelung würde dadurch erheblich steigen.

Die mittlere Periodendauer der spontan-rhythmischen Leberdurchblutungsschwankungen ist mit 34 sec kürzer als die mittlere Schwankungsdauer der Durchblutungsrhythmen in Haut und Skeletmuskel (50—60 sec, BURTON und TAYLOR 1940; 60 sec, GOLENHOFEN und HILDEBRANDT 1957 a), im Rectum und Sigma (30—90 sec, DEMLING und GROMOTKA 1957) und im Uterus (30—60 sec, PRILL 1958) des Menschen. Nach den Untersuchungen von SCHWIEGK (1932) sind in der Leber neben langsameren, durch die V. portae übertragenen Schwankungen auch schnellere Durchblutungsschwankungen im THM-Rhythmus vorhanden, die durch die A. hepatica zugeleitet werden. Die in Abb. 2 dargestellten superponierten Oberwellen könnten so erklärt werden. Ähnliche Oberwellen wurden in der Durchblutung des Rectum und Sigma sowie im Druck der V. portae gefunden (DEMLING und GROMOTKA 1957, GERSMEYER, GASTEYER und GERSMEYER 1957). Es kann vermutet werden, dass nach Abzug der im THM-Rhythmus ablaufenden Schwankungen die mittlere Periodendauer der spontan-rhythmischen Leberdurchblutungsschwankungen bei längeren Zeiten wie die Periodendauern der Schwankungen in den anderen Kreislaufgebieten liegt.

Der Mechanismus der verschiedentlich beobachteten Beeinflussung, Auslösung oder Unterdrückung der spontan-rhythmischen Leberdurchblutungsschwankungen ist unbekannt. Anhaltspunkte liefern Befunde über das Verhalten der Milzvenendurchblutung. Diese zeigt nach Adrenalin, Änderungen des art. Blutdrucks oder O_2-Konzentration in der Atemluft, beim Einschlafen, beim Erschrecken der Versuchstiere und anderen Emotionen periodische Schwankungen von 30—60 sec Dauer, die auch in der Leber messbar sein werden (ROY 1880, SCHWIEGK 1932, BARCROFT, KHANNA und NISIMARU 1932, BARCROFT und NISIMARU 1932, GRINDLAY, HERRICK und BALDES 1939, SEMBA, FUKUBA und KISHI 1951).

GRAYSON (1954 a, b) und GINSBURG und GRAYSON (1954) fanden am narkotisierten Tier mit ähnlicher Methodik eine über

Stunden auffallend konstante Grösse der Leberdurchblutung, welche erst nach Unterbindung der Milz starke Schwankungen aufwies. Die Milzdurchblutung wird deshalb als ein Regulator für die Konstanthaltung der Leberdurchblutung angesehen. Die Autoren meinen hier aber wahrscheinlich grössere Leberdurchblutungsschwankungen. Am narkotisierten Tier wurden auch mit der von uns verwandten Methodik spontan-rhythmische Schwankungen kaum gesehen (GRAF und GOLENHOFEN 1956).

II. Unregelmässige Durchblutungsschwankungen.

Die unregelmässigen Leberdurchblutungsschwankungen konnten in den meisten Fällen als reaktive Schwankungen definiert werden. Womöglich sind sie alle reaktiver Natur, als Folge von Lageänderungen des Körpers, Temperatureinflüssen sowie Schmerzempfindungen, Geräuschen und anderen Emotionen. Die Ergebnisse anderer Autoren unterstützen diese Vermutung. MATTSON (1929) sah bei wachen Hunden, die erschreckt wurden, eine mehrminütige Abnahme des Lebervolumens. REIN (1931) und SCHWIEGK (1932) beschrieben Leberdurchblutungsveränderungen nach thermischen Hautreizen. HOFBAUER (1948), OLERUD (1953), und GERSMEYER, GASTEYER und GERSMEYER (1957) registrierten den Einfluss des intraabdominellen und portalen Druckes, der leicht durch etwa affektiv bedingte höhere Bauchmuskelspannung vergrössert werden kann, auf die Leberdurchblutung. Psychische Affektionen können zur Freisetzung von Adrenalin mit messbarer Wirkung auf die Zirkulation führen (GOLENHOFEN und HILDEBRANDT 1957 b). Reaktive Leberdurchblutungsänderungen wären nur zu vermeiden, wenn die untersuchte Person keinen physischen und psychischen Reizen ausgesetzt wird. Wie schwer dieses offensichtlich zu erreichen ist, zeigen unsere unter weitgehenden „Ruhebedingungen" durchgeführten Versuche, bei denen die unregelmässigen (reaktiven) Durchblutungsschwankungen im Mittel aller quantitativ auswertbaren Versuche 1.8 mal grösser als die spontan-rhythmischen Durchblutungsschwankungen waren. Bei der Beurteilung von Wirkungen bestimmter Massnahmen, etwa der Zuführung von Pharmaka, auf die Leberdurchblutung muss diese „normale" Schwankungsbreite, die im Einzelfall sehr verschieden gross sein kann, berücksichtigt werden. Ein durchblutungsändernder Effekt ist nur dann sicher, wenn sein integraler Wert über den der Ruheschwankungen hinausgeht.

Zusammenfassung.

1. Bei 31 wachen, ruhigliegenden Patienten wurde die lokale Leberdurchblutung mittels einer Wärmeleitsonde während mehrerer Stunden fortlaufend registriert.

2. Die Leberdurchblutung zeigte häufig spontan-rhythmische Schwankungen mit einer mittleren Periodendauer von 34±7 sec. Die mittlere Amplitude dieser Schwankungen betrug etwa 15 % der mittleren Ruhedurchblutung, die maximalen Amplituden erreichten 70 % der mittleren Ruhedurchblutung. Die spontan-rhythmischen Schwankungen konnten in einigen Fällen durch Pharmaka sowie während des Einschlafens verändert, ganz unterdrückt oder auch erst ausgelöst werden.

3. Unabhängig von den spontan-rhythmischen Durchblutungsschwankungen erfolgten häufig unregelmässige Durchblutungsänderungen, die gewöhnlich als Reaktion auf Lageänderungen des Körpers, auf Schmerzempfindungen oder auf Emotionen zu deuten waren. Ihre Amplitude betrug maximal 75 % der mittleren Ruhedurchblutung.

4. Die Möglichkeit eines zeitlichen Zusammenhanges spontan-rhythmischer Durchblutungsschwankungen in der Leber mit spontan-rhythmischen Durchblutungsschwankungen in anderen Kreislaufgebieten wie Skeletmuskulatur, Haut und Mesenterialorganen wird diskutiert.

Summary.

1. Local liver blood flow has been recorded continuously for several hours in 31 human subjects by means of a „Wärmeleitsonde".

2. The local liver blood flow displayed more or less regular spontaneously rhythmical fluctuations with an average frequency of 34±7 sec. The mean amplitude of these variations amounted to 15 per cent of the mean resting blood flow, the maximal amplitudes to 70 per cent. In some instances these spontaneously rhythmical fluctuations disappeared subsequent to the administration of drugs or while the subject was falling asleep, in other cases they appeared when certain agents had been given.

3. Independent of the spontaneously rhythmical fluctuation certain irregular blood flow changes occurred. These were interpret-

ed as secondary to changes in body position, pain or emotional reactions of the patient. Their amplitude amounted to 75 per cent of the mean resting blood flow.

4. The possibility of a time relationship between the spontaneously rhythmical blood flow fluctuations in the liver and similar changes in muscle, skin and mesenteric organs is discussed.

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Methylation of Histamine by Mouse Liver in Vitro.

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Lately it has been shown by SCHAYER and his coworkers that in many species 1-methyl-4-acetic acid is a main metabolic product of histamine. Thus radioactive 1,4-methylImAA[1] has been found in the urine of man, mouse, guinea pig, rabbit, dog and cat following upon injection of labelled histamine, and in man and cat after oral administration (SCHAYER 1956). KOBAYASHI and SCHAYER (1956) have also demonstrated that 1,4-methylImAA is formed, when histamine is incubated with cat liver homogenates. These findings direct the attention to the supposed methylating enzyme system.

As mentioned above the main histamine metabolite found in mouse urine is 1,4-methylImAA. Experiments in vivo (SCHAYER 1953) have demonstrated a pronounced histamine metabolizing activity in the liver of the mouse. Hence it might be inferred that this organ should be very rich in the methylating enzyme system. However, in the mouse liver KOBAYASHI (1957), using a partly

[1] The following abbreviations will be used: ImAA for 4 (5) imidazoleacetic acid; 1,4-methylImAA for 1-methylimidazole-4-acetic acid; 1,4-methylhistamine for 1-methyl-4-(β-aminoethyl) imidazole; N-methylhistamine for exo-N-methylhistamine; IBINH for 1-isobutyl-2-isonicotinylhydrazine; PCA for perchloric acid.

purified extract considered to be free from diamine oxidase activity found the main histamine metabolite to be ImAA. No methylation occurred.

In this communication are reported some experiments, performed in the course of an investigation into the requirements for methylation of histamine, and demonstrating the formation of 1,4-methylImAA and 1,4-methylhistamine in slices of mouse liver.

Experimental.

General Methods. Mice were killed by decapitation, and exsanguinated. The livers were removed immediately, sliced by hand, washed, and placed in Erlenmeyer flasks with 40 μg, 1 μC histamine (2-ring-^{14}C) dihydrochloride and 2 mg methionine in 8 ml of a medium according to KREBS and HENSELEIT (1932). The flasks were incubated at 37° C. Aliquots of the suspension medium were removed at zero time and after 1 and 2 hours. These samples were treated with PCA (for a description see HURLBERT 1954). At the end of the incubation the slices were ground in a blendor and extracted with PCA. All extracts were lyophilized. The residues were dissolved in water, and subjected to paper chromatographic analysis in the following manner.

The samples are spotted together with non-isotopic histamine, 1,4-methylhistamine, ImAA, acetylhistamine, and occasionally isotopic histamine and 1,4-methylImAA. If not stated otherwise, the chromatograms are developed in formic acid, 25 per cent pyridine, and tertiary butanol 15 : 15 : 70, dried, and assayed for radioactivity (FRIESEKE-HOEPFNER, FH 452, methancounter FH 407 with end-window 0.8 mg/cm^2). All figures have been traced from the graphs obtained by the self-registering apparatus, the starting line of the chromatograms being located to the ordinata in origo. The non-isotopic test substances are made visible by diazotization or by dipping in 0.5 % ninhydrine in acetone.

Identification of metabolites. The chromatograms for the suspension medium as well as for the ground slices had one peak corresponding to histamine and a second peak between ImAA and acetylhistamine, the second coinciding with authentic 1,4-methylImAA subjected to the above procedure. When the latter samples were chromatographed together with isotopic histamine

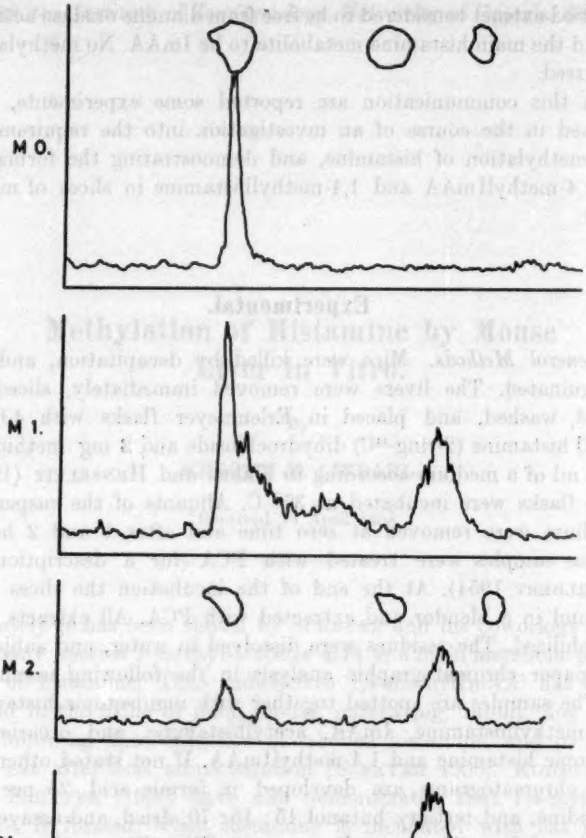

Fig. 1. Paper chromatogram on mouse liver incubated with ^{14}C-histamine: PCA-extracts on suspension medium withdrawn at zero time (MO), after incubation for 1 hour (M1) and 2 hours (M2), and on ground slices after incubation for 2 hours (Ms). Test substances from left to right: histamine, imidazoleacetic acid, and acetylhistamine.

and 1,4-methylImAA, only two radioactive peaks appeared (Fig. 3, a—c). On the chromatograms for the suspension medium a third smaller peak, corresponding to 1,4-methylhistamine, appeared below the histamine spot (Fig. 1, M2).

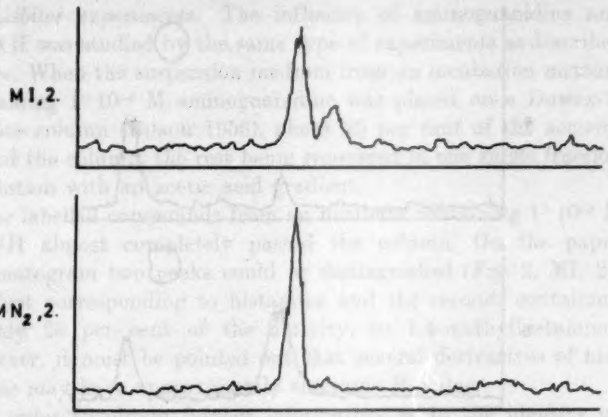

Fig. 2. Paper chromatogram on mouse liver incubated with ^{14}C-histamine. PCA-extracts on suspension medium withdrawn after 2 hours' incubation. Incubation carried out under nitrogen (MN_2, 2) and in the presence of IBINH $1 \cdot 10^{-3}$ M (MI, 2).

Besides the radioactive material the PCA-extracts contained salts and several amino acids which may have interfered with the migration rates. In order to eliminate this source of error the sample, withdrawn after two hours' incubation, was purified on a column of Dowex-1 in formate form (HURLBERT 1954) eluted with formic acid (gradient elution 200 ml $H_2O \rightarrow 1$ M HCOOH, volume of fraction 2 ml). Two radioactive fractions were obtained. The first fraction contained material which was not adsorbed on the column, *i. e.* basic substances as histamine. The activity was too weak to allow further analysis. The second contained roughly 75 per cent of the radioactivity, but no ninhydrine reactive material in concentrations big enough to be detectable on the chromatograms. On the paper chromatogram this fraction coincided with authentic 1,4-methylImAA. After hydrolysis in 1 M HCl for 5 hours at 180° C in sealed tube it still showed the same R_f value, the acid being removed on Dowex-50 prior to chromatography. This material was also chromatographed together with labelled ImAA which was obtained by incubation of ^{14}C histamine with placental extract, and purified on a Dowex-1-acetate column (LINDAHL 1958). Two distinct peaks appeared (Fig. 3 d).

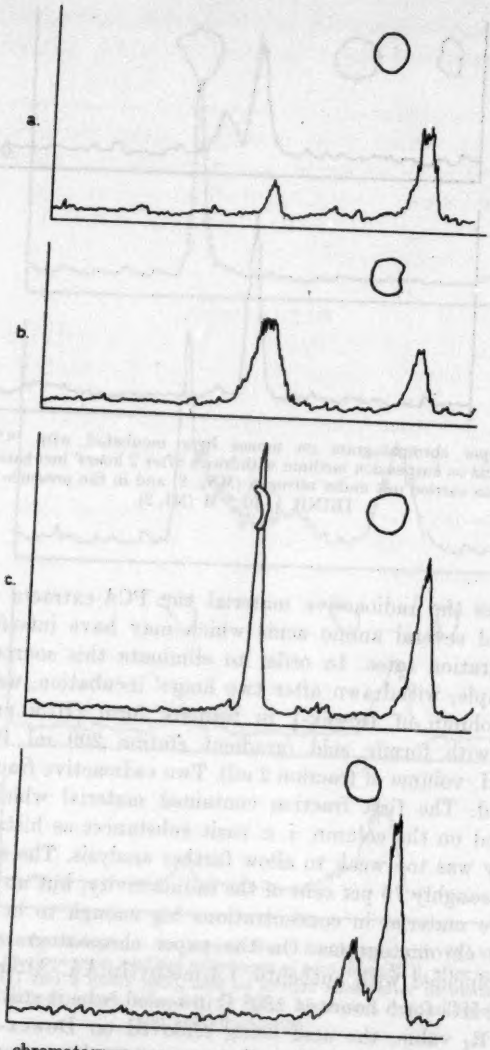

Fig. 3. Paper chromatogram on mouse liver incubated with ^{14}C-histamine. a—c: PCA extracts on slices ground after 2 hours' incubation. Chromatographed together with isotopic histamine and methylimidazoleacetic acid and non-isotopic imidazoleacetic acid: examined sample alone (a); test substances alone (b); all together (c).
d: PCA extract on suspension medium after 2 hours' incubation, purified on Dowex-1, together with biologically labelled imidazoleacetic acid (see text) and non-isotopic imidazoleacetic acid.

Inhibitor experiments. The influence of aminoguanidine and IBINH was studied by the same type of experiments as described above. When the suspension medium from an incubation mixture containing $1 \cdot 10^{-4}$ M aminoguanidine was placed on a Dowex-1-acetate column (BUSCH 1956), about 25 per cent of the activity passed the column, the rest being recovered in one single fraction on elution with an acetic acid gradient.

The labelled compounds from an incubate containing $1 \cdot 10^{-3}$ M IBINH almost completely passed the column. On the paper chromatogram two peaks could be distinguished (Fig. 2, MI, 2), the first corresponding to histamine and the second, containing roughly 20 per cent of the activity, to 1,4-methylhistamine. However, it must be pointed out that several derivatives of histamine may have approximately the same R_f value.

In order to obtain further information as to the identity of the compound small amounts of non-isotopic histamine and 1,4-methylhistamine were added to an aliquot of the PCA-extract. The mixture was saturated with Na_2SO_4, made alkaline, and extracted with chloroform.[1] This material was spotted on chromatograms. A ninhydrine reactive spot corresponding to 1,4-methylhistamine was obtained. On running the chromatograms in three different solvent systems (pyridine, formic acid, t-butanol as above; etanol, 0.1 M HCl 95 : 5; isopropanol, pyridine, water 3 : 2 : 1) the radioactivity was located to the same level as that of the marker. Co-chromatograms with ^{14}C histamine gave two peaks coinciding with the ninhydrine spots for histamine and 1,4-methylhistamine when run in the above solvent systems.

Another aliquot of the same extract was incubated with a diamine oxidase preparation from pig kidney (TABOR 1951, purified as his step A). The incubation mixture was purified on Dowex-1 and thereafter on Dowex-50, and spotted on chromatograms. Two peaks corresponding to ImAA and 1,4-methylImAA appeared.

Oxygen requirement. In one experiment the incubation of slices of mouse liver was carried out under nitrogen. In this case the histamine did not seem to be metabolized to any appreciable extent (Fig. 2, MN_2, 2).

Homogenates. Homogenates on mouse liver did not produce any essential histamine degradation.

[1] ROTSCHILD and SCHAYER, unpublished method referred by Lindell, S.-E., Dissertation Lund 1958.

Discussion.

The above experiments prove with satisfactory certainty the identity of the final histamine metabolite in mouse liver slices with 1,4-methylImAA. The metabolite has the same migration rate as authentic 1,4-methylImAA, the compounds appearing as one single spot on a paper chromatogram. In the same solvent system the metabolite separates well from ImAA. It is not destroyed by hydrolysis, and is thus distinguished from acetylhistamine and ImAA-riboside.

The identification of a prior intermediary metabolite as 1,4-methylhistamine must also be regarded satisfactory, since the investigated substance moves together with authentic 1,4-methylhistamine in three different solvent systems. The fact that 1,4-methylImAA is formed by the action of diamine oxidase rules out N-methylhistamine, reported to be a histamine metabolite *in vivo* (KAPELLER-ADLER 1957, LINDELL and WESTLING, 1957).

Identification of the metabolites was the primary aim of this investigation. Hence the methods used allow only a rough determination of quantitative ratios. However, as the results are very distinct, some conclusions can be drawn. Thus the inhibitor experiments indicate that histamine metabolism in mouse liver is influenced very markedly by IBINH, but not by aminoguanidine. In the presence of IBINH, methylation of histamine proceeds to a certain extent, but the subsequent oxidation is blocked. Evidently the latter reaction is not catalyzed by diamine oxidase, this enzyme being very susceptible to aminoguanidine. It might be inferred that the oxidizing enzyme is identical with the amine oxidase in dialyzed extracts from mouse liver, observed by KOBAYASHI (1957), provided that the non-formation of ImAA is attributed to special properties of the intact cell.

The incubations carried out in a nitrogen atmosphere indicate that also the methylation process is aerobic, which suggests a relation to the energy generating mechanisms.

Summary.

The exposed experiments give evidence that the histamine degradation in mouse liver is achieved by the methylating enzyme system, demonstrated *in vivo* by SCHAYER. Thus the main histamine metabolite found in slices is 1-methylimidazole-4-acetic

acid. Furthermore, the histamine destruction is largely resistant to aminoguanidine which is a strong diamine oxidase inhibitor. Isobutyl-isonicotinylhydrazin, a monoamine oxidase inhibitor, prevents the formation of 1-methylimidazole-4-acetic acid, but does not altogether exclude the formation of 1-methyl-4-(β-aminoethyl)imidazole. These characteristics are to be expected for the methylating enzyme system on account of recent findings *in vivo* (SCHAYER and KARJALA 1956).

The author is greatly indebted to Dr. R. SCHAYER for supplying a sample of labelled 1-methylimidazole-4-acetic acid.

Dr. H. TABOR has supplied a method for preparing imidazoleacetic acid, and also provided a sample which is hereby gratefully acknowledged.

Samples of isonicotinyl-isobutylhydrazin and 1,4-methylhistamine were generously donated by Drs. WESTLING and LINDELL.

The Frieseke-Hoepfner apparatus was made available through the courtesy of Prof. J. BÖÖK.

To Prof. P. E. LINDAHL I wish to express my particular indebtedness for the kind interest he has shown in my work.

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The Effect of Starvation on the Phagocytic Activity of the Reticulo-Endothelial System.

By

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The functional activity of the reticulo-endothelial system (R. E. S.) is usually measured by studying its ability to eliminate injected particulate matter from the blood stream. Most of the particles injected are taken up by the RE-cells of the liver. If insufficient amounts of particles are injected the liver might take up all the particles passing it in one passage and thus their elimination will be a function of the portal blood flow. To afford a measure of the phagocytic activity of the RE-cells, however, the particles must be administered in large amounts (BENACERRAF *et al.* 1957). Another factor of importance in the study of phagocytic activity is to have homogeneous and stabile suspensions (DOBSON 1957). Suitable inert spherical particles filling these requirements have been prepared by emulsion-polymerization of methyl methacrylate (JUHLIN 1956). Inanition or starvation in preliminary experiments was also found to be a factor which might influence the results in studies of R. E. S. function. This investigation was initially started to study the sources of error that could be caused by starvation.

In starved animals the weight of the liver is greatly decreased. The size of the liver-cells as well as of the basophil cytoplasmic inclusions and nucleoli is decreased (LAGERSTEDT 1949). WEBER and CANTERO (1957) found in animals starved during 24 hours

that the amount of liver-cells/g wet weight was increased. Little is known, however, of what happens to the RE-cells during acute starvation.

Of interest in this connexion are the experiments with radioactive gold colloids by DI LUZIO (1957), who suggested that chylomicrons could physiologically activate the R. E. S. During starvation there would be no such activator of the R. E. S. and one might therefore expect a decreased R. E. S. activity. FREUND (1932), however, found that fasting during 24 hours leads to an increased deposition of injected colloidal particles (gold and saccharated iron) in the Kupffer cells, while the amount of particles was decreased in animals receiving large amounts of sugar per os. TIMIRAS (1953), using Indian ink, and DI LUZIO (1956), using colloidal gold, found an increased uptake of the particles in the lungs of rats starved during 2—4 days, but no change in the total liver uptake. In animals with restricted food intake during 28 days, GORDON and KATSH (1952) found an increased uptake of colloidal thorium by the spleen. The discrepancies might be due to the different methods used, which in one or several points are open to criticism. It was thus thought worth while further to study the effect of starvation on the phagocytic activity of the R. E. S.

Methods.

Test animals.

Male albino rabbits weighing 1.95—2.05 kg were used. They were fed on hay, oats and water *ad lib.* During a certain period before the experiment some animals were starved and given only water.

Particles.

Spherical particles of methyl methacrylate plastic, marked with a fluorescent dye, 'Fluorescent Green H. W. 185 %' (Wilmot & Cassidy Inc., N. Y.) were used. These were from the same batch as described earlier (JUHLIN 1956), where the diameter of the particles was about 0.1 μ and their calculated HLB (hydrophilic-lipophilic balance) value about 40. As evident from electronmicroscopic photos and the size distribution table (JUHLIN 1956) the particles are of a rather uniform size.

Doses injected: From the original particle suspension, which contained 24 % by weight of solids, 1 ml was added to 14 ml of 5 % sterile glucose solution. Of this solution 6 ml was injected intravenously 2 times with an interval of one hour. Each rabbit thus received 192 mg of solid particles.

Experimental procedure:

The unanaesthetized rabbits were placed in a box with their necks fixed in an aperture. From an injection needle inserted in the middle artery of the pinna blood could be rapidly withdrawn at intervals. The needle was fixed with adhesive tape and left in situ with a little metal stopper instead of the syringe when no blood was being withdrawn. To prevent coagulation 10 mg heparin (Heparin Vitrum 1 %) was injected i. v. prior to the experiment.

Into the pinna not used for blood sampling 6 ml of the particle suspension was injected intravenously during 2 minutes. Immediately after injection and at regular intervals 1 ml arterial blood was collected and added to 10 ml of pure acetone. After 1 hour an additional 6 ml of the particle suspension was injected i. v. and blood collected as before. Altogether about 24 ml blood was removed. Three hours after the first particle injection the animals were stunned by a blow on the neck and both carotid arteries were opened to bleed the animal to death. Internal organs were then removed and wet-weighed. The particles were found to be localized in the liver, spleen, lung and bone marrow. The particle distribution in these organs and the blood was estimated as described hereafter.

Estimation of particle fluorescence.

The organ to be examined was cut in very fine pieces with scissors and stirred to a porridge-like mass. From this 0.5 g or usually 1.0 g was added to 10 ml of acetone. The mixture was then shaken several times during 2 hours. This proved quite sufficient to dissolve the particles and their fluorescent dye. The mixture was centrifuged and the clear acetone solution was decanted into Coleman 12—190 cuvettes. The same procedure was used for blood where 1 ml had been added to 10 ml acetone.

The fluorescence was determined in a Coleman Electronic Photofluorometer with filter 12—222 between lamp and sample and filter 14—212 between sample and phototube. Solutions with a known content of particles were used as standards. In both starved and control animals, injected with non-fluorescent particles, practically no fluorescence was found in blood, bone marrow, spleen and lung. Some fluorescence was found to be fairly constant and corresponded to a mean of 168 ± 24 μg particles/g liver tissue. The figures for particle amounts in the liver have been corrected for this blank value.

Estimation of particles in bone marrow.

All marrow found in one femur of each animal (0.7—1.5 g) was taken out and the amount of particles/g was measured. No significant difference in weight of bone marrow was found between starved and control animals. In preliminary experiments all bones in the bodies of 5 animals were examined for their content of particles (JUHLIN, unpublished). In most bones it was not possible to estimate the weight of the marrow. If, however, the amount of particles/g marrow in all bones is assumed to be the same as in the femur one obtains an expression for the total

amount of femur-equivalent bone marrow. This amount was calculated to be 30 g in a 2 kg rabbit. In the present experiments it has been assumed that all the bone marrow undergoes the same changes as that of the femur.

Phagocytic index and method of combining results from different experiments.

The clearance of particles from the blood stream is largely in accordance with an exponential equation $(\log C_1 - \log C_2)/(T_1 - T_2) = K$, where C_1 and C_2 are the concentrations of particles in mg/100 ml blood at the times T_1 and T_2 respectively. K is a constant representing the rate of clearance of particles from the blood and called the phagocytic index (BENACERRAF *et al.* 1957). The phagocytic index has been calculated both after the first particle injection (K_1) and after the second injection (K_2). If the values for K are divided by $\log \frac{100}{99} = 0.0044$ we get the percentage drop in particle concentration per minute.

The geometric means of the observed particle concentrations at various time intervals after injection were used for the construction of the average curves in Fig. 1. This procedure ensures that the rectilinearity on semilogarithmic paper of the individual experimental curves is not destroyed by the pooling of the data.

The K-values in Table I are arithmetical means of individual K-values obtained by plotting each individual experiment on semilog paper and visually measuring K from the straight portion of the curve. This average K agrees closely with the K of the straight parts of Fig. 1 obtained by a different but equivalent arithmetical procedure.

Results.

The results are summarized in Table I and Fig. 1—4. The following facts are established:

1. Intravenously injected particles disappeared more slowly from the blood stream in rabbits starved during 4 days than in control rabbits. The rate of clearance varied during the first minutes after injection, especially in starved animals, and then became constant (Fig. 1).

2. Starvation during 4 days reduced the weight of the liver and spleen by about 30 %. No significant decrease was found in the bone marrow of the femur or in the lungs.

3. Starvation decreased the fraction of the total amount of particles phagocytized by the liver but increased the amount of particles in the bone marrow. In the spleen and the lungs the changes in the total amount of particles were not significant.

4. In 4-day-starved rabbits the amount of particles/g tissue was

Table I.

Distribution of spherical particles in certain organs of 2 kg male rabbits. Injected dose of particles 192 mg. All organ analyses 2 hours after the last intravenous injection.

The figures given in I and II indicate arithmetic means and ranges of the observations. The figures in column III and IV indicate arithmetic mean ± the standard error of the mean.

	I	II	III	IV
	Starved 24 hours	Starved 4 days and then given food 24 hours before test	Starved during 4 days	Controls
Blood				
Percentual drop in particle concentration per minute ..... Inj. I	7.5 (5.0—11.4)	5.7 (5.5—5.9)	5.7±0.7	10.0±0.7
Inj. II	3.9 (2.7—9.5)	3.4 (3.0—4.1)	3.4±0.5	7.7±0.7
K_1 According to Biozzi *et al.* 1954	0.033 (0.022—0.050)	0.025 (0.024—0.026)	0.025±0.003	0.044±0.00
K_2	0.017 (0.012—0.042)	0.015 (0.013—0.018)	0.015±0.002	0.034±0.00
Liver				
weight g	65.0 (59—80)	68.6 (45—96)	44.4±1.1	66.4±5.4
mg particles/g	1.7 (1.5—2.0)	1.6 (0.9—1.9)	2.2±0.1	2.0±0.1
% of injected dose ...	59.4 (53—63)	52.4 (46—57)	50.7±2.5	66.9±1.6
Spleen				
weight g	1.0 (0.7—1.1)	0.7 (0.6—0.8)	0.6±0.06	1.1±0.2
mg particles/g	10.1 (8.0—12.0)	9.2 (5.9—11.8)	12.6±1.4	10.4±1.3
% of injected dose ...	5.8 (3.0—8.6)	3.2 (1.6—4.5)	4.1±0.6	5.8±0.6
Bone marrow				
weight g	30.0	30.0	30.0	30.0
mg particles/g	1.5 (1.0—2.1)	2.4 (2.1—2.7)	2.5±0.2	1.6±0.1
% of injected dose ...	28.0 (20—38)	38.0 (33—42)	38.9±2.6	24.8±1.6
Lung				
weight g	9.6 (7.4—10.2)	9.0 (8.0—11.0)	8.5±0.7	9.2±0.5
mg particles/g	0.5 (0.4—0.5)	0.7 (0.6—0.9)	0.6±0.2	0.4±0.1
% of injected dose ...	2.7 (1.8—4.5)	2.8 (1.4—4.2)	3.2±1.2	1.9±0.2
Body weight kg	[1]1.99 [2]1.92	[2]2.04 [2]1.76	[1]1.99±0.04 [2]1.67±0.03	2.00±0.03 —
Number of animals	4	4	9	9

[1] Before starvation
[2] After »

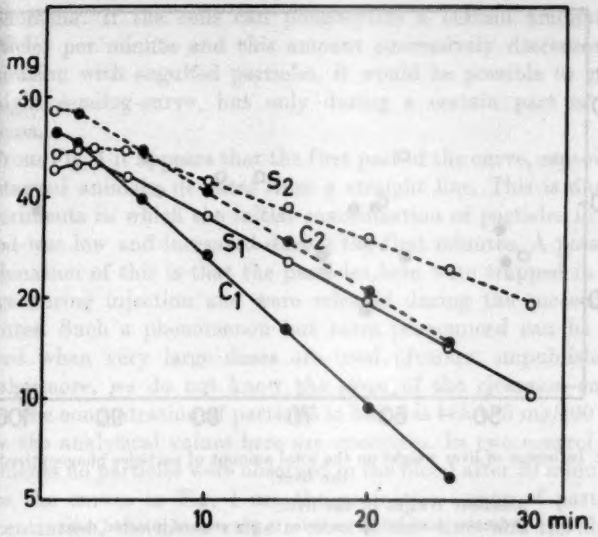

Fig. 1. Clearance of particles from the blood of 4-day-starved rabbits (S) and controls (C). The same dose 96 mg was given twice with an interval of 1 hour (subscripts 1 and 2). The initial body weight of all animals was 2 kg.

Abscissa: Time in minutes after injection of particles.
Ordinate: Particles, mg/100 ml blood.

increased in the femur bone marrow whereas no differences from the controls were found in the liver, spleen and lung.

5. In animals starved during 4 days and given food 24 hours before the experiment the particles were distributed and cleared as in animals starved only.

6. In rabbits deprived of food during 24 hours the phagocytic index in some experiments decreased as in starved animals, and the percentage of particles found in the liver tended to be slightly decreased.

7. The amount of particles/g liver in control animals is less in a large liver than in a small one. This causes the fraction of injected particles found in the liver to show less dispersion than the weight of the liver (Fig. 2). A similar tendency is noticeable in animals starved during 24 hours and in 4-day-starved animals given food 24 hours before the test.

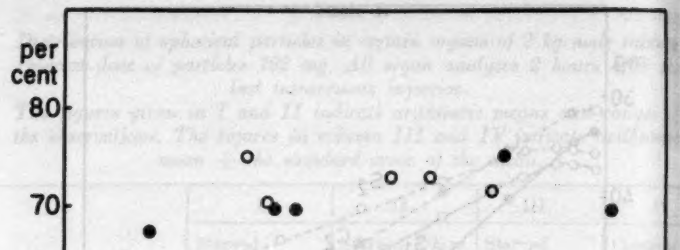

Fig. 2. Influence of liver weight on the total amount of particles phagocytized by the liver.

Abscissa: Weight of the liver.
Ordinate: Amount of particles in per cent of injected dose.

● Control animals (Table I) where blood had been sampled for estimation of K.
○ Control animals not bled before death.

Discussion.

Clearance of particles from blood.

When the time-concentration curve of particles in blood is plotted on a semilogarithmic grid (Fig. 1) we find that some minutes after injection a straight line results, indicating that a constant percentage of particles is eliminated per unit of time independent of the blood particle concentration. The slope of this line has been called the phagocytic index (page 265). It is improbable, however, that the various factors affecting the rate of removal of particles can readily be condensed into one parameter, the phagocytic index, even if during part of the process the shape of the concentration curve suggests the existence of this very simple situation.

Biozzi, Benacerraf and Halpern (1953) found that the phagocytic index decreased on repeated injections even of small doses. Similar results with larger doses were obtained in the present experiments. They definitely indicate the existence of saturation

phenomena. If the cells can phagocytize a certain amount of particles per minute and this amount successively decreases by saturation with engulfed particles, it would be possible to get a straight semilog-curve, but only during a certain part of the process.

From Fig. 1 it appears that the first part of the curve, especially in starved animals, deviates from a straight line. This is due to experiments in which the initial concentration of particles in the blood was low and increased during the first minutes. A possible explanation of this is that the particles here were trapped in the lungs during injection and were released during the succeeding minutes. Such a phenomenon but more pronounced can be observed when very large doses are used (JUHLIN, unpublished). Furthermore, we do not know the slope of the clearance-curve when the concentration of particles in blood is below 5 mg/100 ml, since the analytical values here are uncertain. In two control experiments no particles were observed in the blood after 30 minutes. Since the curves in Fig. 1 are the geometric means of particle concentration, the mean value is zero at this time and the slope infinitely large. Thus the rectilinearity on a semilog-plot does not seem to persist during the whole phagocytic process.

The clearance of particles from the blood is known to be dependent upon several factors (BIOZZI *et al.* 1953, JANSCÓ 1956, DOBSON 1957). The sum of their effects, the phagocytic index (K), is approximately constant during a relatively long time. Its nature is uncertain, but, provided certain factors are constant, it might be taken as a measure of phagocytic activity. It is necessary to remember the aforementioned limitations, however.

Role of the liver in the phagocytic activity of the R. E. S.

The total amount of particles found in the liver of starved animals was decreased. The question arises: was this because the amount of RE-cells in contact with blood had decreased and/or because the phagocytic activity of the RE-cells had decreased.

Concerning the first part of the question, we know that a decrease in the weight of the liver does not change its total number of cells (WEBER and CANTERO 1957) and that the number of Kupffer cells per hepatic cell remains unchanged (GORDON and KATSH 1952). Hence the decreased amount of particles found in the liver of starved animals is not due to a decrease in the amount of Kupffer-cells, but it might depend upon decrease in the amount

of blood passing the RE-cells. Di Luzio and Powers (1956), however, found no change induced by starvation in rats in the removal rate of intravenously injected colloidal Au^{198}. The intravascular half-life time of Au^{198} was found to be from 54—92 sec (Di Luzio 1956). In these experiments the clearance probably was not a measure of phagocytic activity but of the portal blood flow which remained unchanged. Since thus starvation does not change the number of Kupffer-cells or the portal blood flow it is probable that the decreased phagocytic index is due to a decreased phagocytic activity. As is apparent from the distribution values discussed later, it is primarily the RE-cells of the liver that decrease their phagocytic activity.

Benacerraf *et al.* (1957) found variations in phagocytic index in mice and rats, which were shown to be due to differences in the relative size of the liver and spleen. Rabbits and guinea pigs showed much smaller variations. They introduced for rats and mice a corrected phagocytic index $\alpha = {}^{w}/_{w_{ls}} \times \sqrt[3]{K}$, where w is the weight of the individual animal and w_{ls} the weight of the liver and spleen. To investigate the same correlation in rabbits the phagocytic index has been correlated with the weight of the liver (Fig. 3—4). In rabbits the variations in the weight of the spleen are small compared with those in the weight of the liver and have therefore been disregarded. The body weight was considered practically constant since its decrease in starved animals was mainly due to loss of gastro-intestinal contents. From Fig. 3 and 4 it is evident that the phagocytic index (K) increases with an increase in the weight of the liver but that the dispersion for K_1 is great. Hence any mathematical expression of this correlation is difficult to set up. It does not seem to be the same as found for rats and mice by Biozzi *et al.* (1953). The phagocytic index in animals starved during 4 days is lower, however, than one would expect from corresponding decrease in liver weight in control animals (Fig. 4). If the starved animals were given food 24 hours before the test, the liver weight seemed to increase without any corresponding increase in phagocytic activity. In animals deprived of food during 24 hours the phagocytic index seemed to decrease before any corresponding decrease in liver weight could be observed. Thus the decreased phagocytic activity in starved animals does not seem to be due to a decrease in liver weight.

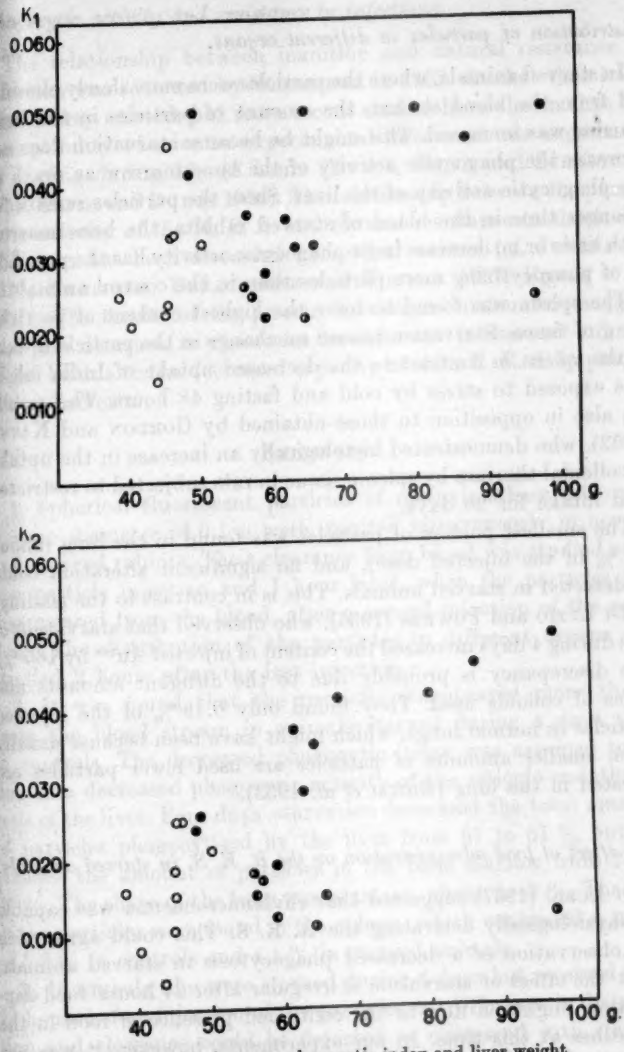

Fig. 3 and 4. Relationship between phagocytic index and liver-weight.

Figure 3 after the first particle injection (K_1).
Figure 4 after the second particle injection (K_2).
○ Animals starved during 4 days.
◑ Animals starved during 4 days and given food 24 hours before the experiment.
◐ Animals starved during 24 hours.
● Controls.

Distribution of particles in different organs.

In starved animals, where the particles were more slowly eliminated from the blood stream, the amount of particles in the bone marrow was increased. This might be because starvation does not decrease the phagocytic activity of the bone marrow as much as the phagocytic activity of the liver. Since the particles remain for a longer time in the blood of starved rabbits, the bone marrow with little or no decrease in its phagocytic activity has the possibility of phagocytizing more particles than in the control animals.

The spleen was found to have the highest content of particles per g of tissue. Starvation caused no change in the particle uptake of the spleen in contrast to the decreased uptake of India ink in rats exposed to stress by cold and fasting 48 hours. The results are also in opposition to those obtained by GORDON and KATSH (1952), who demonstrated histologically an increase in the uptake of colloidal thorium by splenic tissue in rats subjected to restricted food intake for 28 days.

The smallest portion of particles was found in the lung (about 2 0 % of the injected dose), and no significant alteration could e detected in starved animals. This is in contrast to the findings of DI LUZIO and POWERS (1956), who observed that starvation of rats during 4 days increased the content of injected Au^{198} by 165 %. The discrepancy is probably due to the different amounts and types of colloids used. They found only 0.19 % of the injected particles in normal lungs, which might have been because usually when smaller amounts of particles are used fewer particles are arrested in the lung (BIOZZI *et al.* 1953).

The effect of food administration on the R. E. S. in starved animals.

DI LUZIO (1957) suggested that chylomicronaemia was capable of physiologically activating the R. E. S. This could agree with the observation of a decreased phagocytosis in starved animals. That the effect of starvation is irregular after 24 hours' food deprivation might be due to the continued presence of food in the intestines at this time. In our experiments, however, it was not possible to activate the R. E. S. in starved animals by giving them food 24 hours before the test. In any event, food intake might be a factor of importance in explaining effects on the phagocytic activity of the R. E. S.

Phagocytic activity and resistance to infections.

The relationship between inanition and natural resistance to infection was reviewed by SCHNEIDER in 1952. At that time some controversy existed, but more recent studies have shown that the susceptibility of mice to experimental infections was augmented by fasting during 36—48 hours (DUBOS, SMITH and SCHAEDLER 1955, SMITH and DUBOS 1956). The relationship between the phagocytic activity of the R. E. S. and natural resistance to shock and infections has been discussed by several writers (*cf.* JUNGEBLUT 1930, ZWEIFACH, BENACERRAF and THOMAS 1957, BIOZZI *et al.* 1957). The results of the present investigation therefore suggest that the decrease in resistance to infection in starved animals might be due to the decreased capacity of their R. E. S. to phagocytize bacteria.

Summary.

1. Spherical fluorescent particles of methylmethacrylate with a mean diameter of 0.1 μ were injected intravenously in normal and starved rabbits. Their clearance from blood was studied after one particle injection and 1 hour later, when the particles had disappeared from the blood, after a second injection of the same dose. The distribution of the particles in different organs was studied 2 hours after the last injection.

2. It was found that the particles disappeared more slowly from the blood stream in animals starved during 4 days than in controls. The decreased phagocytic index was assumed to be due to a decreased phagocytic activity of the reticulo-endothelial cells of the liver. Four days' starvation decreased the total amount of particles phagocytized by the liver from 67 to 51 % but increased the amount of particles in the bone marrow from 25 to 39 %. The share of the lung was constant, about 2—3 %. The rest of the particles were found in the spleen, which contained a mean of 5.8 % in controls and 4.1 % in starved animals.

3. If animals who were starved during 4 days but received food 24 hours before injection of the particles, no difference in distribution and clearance could be detected as compared with starved animals. After only 24 hours food deprivation irregular results were obtained.

4. The mechanism of phagocytosis and the results obtained are discussed. The possibility is suggested that the decreased

resistance to infection in starved animals might in part be due to the decreased ability of their reticulo-endothelial cells to phagocytize bacteria.

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The Effect of Zinc and Certain Other Bivalent Metal Ions on the Isometric Tension Development of Glycerol-extracted Muscle Fibre Bundles.

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In addition to magnesium (~ 10 mmoles/kg, HAMM 1955, EDMAN unpublished) and calcium (~ 1 mmole/kg, HAMM 1955, EDMAN unpublished), striated muscle contains zinc in a rather high concentration (~ 0.8 mmole/kg, KOGA 1934, HAMM 1955, KOCH *et al.* 1956, EDMAN unpublished).[1] While the effects of calcium and magnesium on the contractile mechanism have been extensively investigated, no thorough investigation has, up to the present, been performed with zinc. The present work comprises a comparative study of the effects of zinc and certain other bivalent metals on the isometric ATP-induced contraction of glycerol-extracted muscle fibre bundles. The investigation has also shown that certain metals can produce a reversible relaxation of fibre bundles previously contracted by adenosinetriphosphate (ATP). The relaxing effect of zinc in the presence of ATP was described earlier in a preliminary report (EDMAN 1956).

[1] The values represent content per kg of wet tissue.

Methods.

Fibre bundles from rabbit psoas muscle were used. The extraction and preparation of these bundles as well as the technique for isometric recording of the tension development were described in an earlier publication (EDMAN 1957). The length of the fibre bundles was 10 mm; the cross section was 75—159 × 131—281 μ.

All the substances studied were dissolved in a KCl solution with pH 7.30 containing 100 mM K, 10 mM veronal (diethylbarbituric acid) and 1 mM Mg. Hereafter this solution will be called the "veronal buffer". In the control experiments the veronal buffer alone was used as medium. In the cases of manganese, cobalt and mercury a new stock solution was prepared for each day's experiments; for the rest of the metals the stock solution was used for two weeks at most.

A 0.2 mM stock solution of mercury was prepared by dissolving $HgCl_2$ in bidistilled water and subsequently diluting 50 times with the veronal buffer. The pH of the stock solution was 7.2.

The water used in preparing the solutions and washing the glass vessels had been twice distilled in borosilicate glass. The pH determinations were performed with a glass electrode.

The pH of the final solutions was checked, being allowed to range from 7.20 to 7.30. For the highest concentrations of cadmium (> 1 mM) studied it was necessary to adjust the pH with KOH.

Veronal, diethylbarbituric acid, crystal, Ph. S. XI was used. All other chemicals used were of pro analysi quality. According to an analysis performed by the Analytical Department of the University of Uppsala, one gram of the diethylbarbituric acid substance contained 180 μg ash, 40 μg sodium, 4 μg calcium, < 3 μg magnesium and ≤ 0.5 μg zinc.

ATP was obtained in the form of dibarium salt, "chromatographically pure", from Schwartz Laboratories, Lot no. 5204, and Pabst Laboratories, Lot no. 129. The purity of the ATP preparations and their concentration of ATP, ADP and AMP were checked as described earlier (EDMAN 1957). According to this analysis the amount of total nucleotide in 51 mg of the substance was:

Schwartz, Lot no. 5204: 0.0546 mmole.

Pabst, Lot no. 129: 0.0573 mmole.

The relative nucleotide concentration was as follows:

Schwartz, Lot no. 5204: ATP 89 %, ADP 4 %, AMP traces only, and an unknown fraction, possibly uridine nucleotide, 1 % at most. Pabst, Lot no. 129: ATP 92 %, ADP 2 % and AMP traces only.

The potassium salt of ATP was prepared from Ba_2ATP for each day's experiments. After 92 mg of the Ba_2ATP substance was suspended in 5 ml of bidistilled water, 1 N HCl was added drop by drop until all of the substance had dissolved. Then 1.50 ml of K_2SO_4 solution (25.15 mg/ml) was added. After centrifugation an additional drop of K_2SO_4 solution was added to the ATP solution to ensure a slight excess of SO_4^{--}. Finally, the ATP solution was neutralized to pH 7.3 with 1 N KOH and diluted with the veronal buffer to 60 ml.

In those series of experiments where the effects of metal ions on the course of the *contraction* were studied, control and test experiments were performed alternately. The fibre bundles for the controls and tests were dissected from the same muscle preparation and were treated throughout in a similar manner. After the fibre bundle had been prepared and fastened between the two platinum loops as described earlier (EDMAN 1957), it was placed in a Petri dish containing 60 ml of the control or test solution for two hours immediately before the start of the experiment. This solution was used for 16 fibre bundles at most. The volume of the bath solution during the recording of the contraction was always 40 ml. The contraction was induced by exchanging the glass vessel with bath solution for another containing the same solution, but with ATP added. Not more than 8 experiments were performed with the same bath solution. The initial tension of the fibre bundle was 17.2 mg. The details on the recording of an isometric contraction are given in an earlier publication (EDMAN 1957).

The fibre bundles used in the studies of the *relaxing* effect of metal ions were washed for about one hour after dissection in veronal buffer only. The fibre bundles were given an initial tension of 22.2 mg and were contracted by ATP (0.37—0.40 mM) in a bath of the veronal buffer solution. After 2—5 min., when the contraction was almost finished, the vessel with bath solution was exchanged for another containing the test solution with ATP (0.37—0.40 mM). The volume of the bath solution was 40 ml in the relaxation experiments also. For the data on the concentrations of the metals studied, see Results.

In order to study the relaxing effect of the metal in the absence of ATP the fibre bundle was first immersed in ATP-free veronal buffer solution for 10—15 min. after a contraction. The tension decreases somewhat within the first 5 to 10 min. after removal of the ATP from the bath.

For all experiments presented in this paper the deviation from strict isometry in the recording device was less than 0.06 mm, *i. e.* less than 0.6 % of the length of the fibre bundles.

The experiments were performed at room temperature, 20—22° C.

If not otherwise stated, the data on the concentrations refer to the total concentration in the bath solution.

Results.

I. *The effect of metal ions on the isometric contraction of glycerol-extracted muscle fibre bundles.*

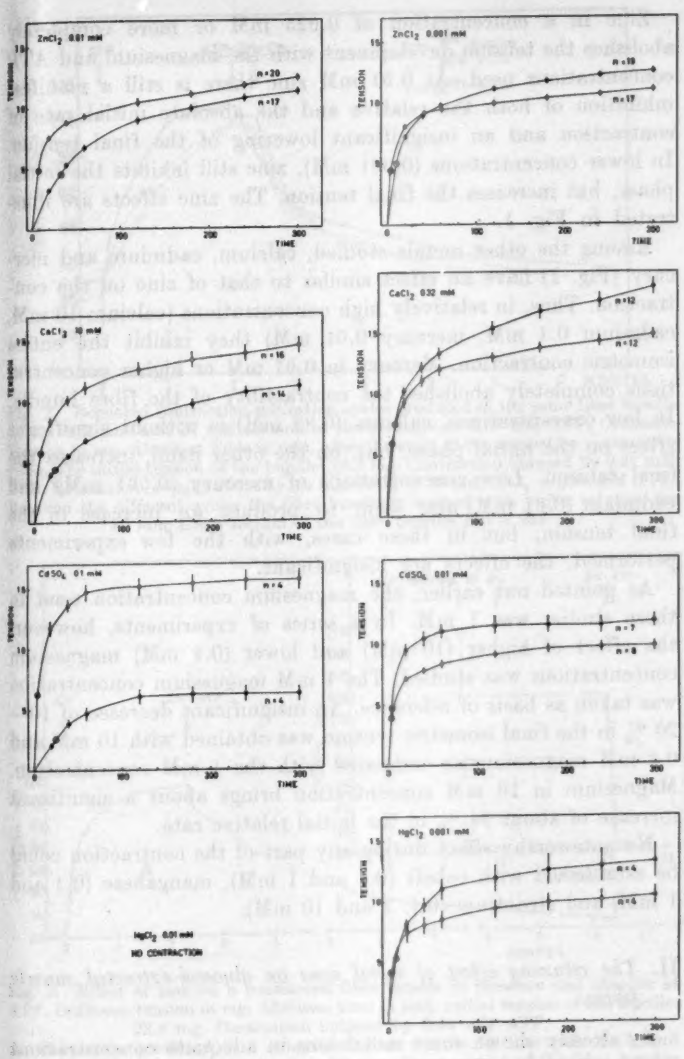
The contraction was induced by 0.22—0.24 mM ATP. The effects of the metals on the entire course of the contraction were studied. Some results are illustrated in Fig. 1. This figure shows the tension developed after different intervals from the start of contraction.

Since the cross section differs from bundle to bundle and ATP can only penetrate into a peripheral layer of the fibre bundle, the tension developed is expressed in relation to the circumference of the fibre bundle. As shown earlier (EDMAN 1957) there is a direct proportionality between the tension developed and the circumference of the fibre bundle under the given conditions. Five minutes after the start of contraction the tension attains a practically constant level. The last values in the figures may therefore be assumed to represent the final tension in the fibre bundle. The slope of the contraction curve is an expression of the "*absolute* rate of contraction" (EDMAN 1957), defined as tension increase per unit of circumference and unit of time. Each figure also shows the time required to reach 40 % of the final tension for test and control experiments respectively.[1] The reciprocal of this time value is an arbitrary measure of the "initial *relative* rate of contraction" (EDMAN 1957), *i. e.* a measure of the fraction of the final tension developed per unit of time during the initial phase of the contraction. The relative rate was used in order to compensate for changes in the rate of contraction brought about by a mere change in the contractility (EDMAN 1957). The "initial *absolute* rate of contraction", as used hereafter, is the mean tension increase per unit of fibre bundle circumference and unit of time over the interval 0—40 % of final tension. It is an approximation to the initial tangent of the curve.

Table I summarizes the results observed in the study of the effects of the metal ions on the initial relative and absolute rates of contraction and on the final isometric tension of the fibre bundle.

[1] The original contraction curve contains small irregularities in the rapid, initial phase. Thus, in order to get a more accurate value for the time required to reach 40 % of the final tension this value was calculated for each experiment as the arithmetical mean of the times for reaching 30, 40 and 50 % of the final tension.

Fig. 1. Effect of high and low concentrations of zinc, calcium, cadmium and mercury on the isometric contraction of glycerol-extracted muscle fibre bundles. ATP concentration 0.22 mM. Cross section of the fibre bundles: 75—159 × 131—281 μ. Ordinate: tension in mg per 100 μ of fibre bundle circumference. Abscissa: time in sec. after immersion in ATP. Filled circles: test experiments, open circles: control experiments, both representing the mean tension developed after different time intervals. The standard error of the mean is indicated with a bar. The number of statistical units is indicated at the respective curves. The average time for reaching 40 % of the final tension is indicated with ⊗ for control curves, and with ⊙ for test curves. The standard error of these latter values does not exceed the size of the symbols.


ZnCl2 0.01 mM
n=20
n=17
ZnCl2 0.001 mM
n=19
n=17
CaCl2 10 mM
n=16
n=14
CaCl2 0.32 mM
n=12
n=12
CdSO4 0.1 mM
n=4
n=4
CdSO4 0.01 mM
n=7
n=8
HgCl2 0.01 mM
NO CONTRACTION
HgCl2 0.001 mM
n=4
n=4
TENSION
TIME

Zinc in a concentration of 0.025 mM or more completely abolishes the tension development with the magnesium and ATP concentrations used. At 0.01 mM zinc there is still a manifest inhibition of both the relative and the absolute initial rate of contraction and an insignificant lowering of the final tension. In lower concentrations (0.001 mM), zinc still inhibits the initial phase, but increases the final tension. The zinc effects are illustrated in Fig. 1.

Among the other metals studied, calcium, cadmium and mercury (Fig. 1) have an effect similar to that of zinc on the contraction. Thus, in relatively high concentrations (calcium 10 mM, cadmium 0.1 mM, mercury 0.01 mM) they inhibit the entire isometric contraction. Mercury in 0.01 mM or higher concentrations completely abolishes the contractility of the fibre bundle. In low concentrations, calcium (0.32 mM) is without significant effect on the initial phase, but, on the other hand, increases the final tension. Low concentrations of mercury (0.001 mM) and cadmium (0.01 mM) also seem to produce an increase in the final tension, but in these cases, with the few experiments performed, the effects are insignificant.

As pointed out earlier, the magnesium concentration used in these studies was 1 mM. In a series of experiments, however, the effect of higher (10 mM) and lower (0.2 mM) magnesium concentrations was studied. The 1 mM magnesium concentration was taken as basis of reference. An insignificant decrease of 10—20 % in the final isometric tension was obtained with 10 mM and 0.2 mM magnesium as compared with the 1 mM concentration. Magnesium in 10 mM concentration brings about a significant increase of about 94 % in the initial relative rate.

No noteworthy effect during any part of the contraction could be established with cobalt (0.1 and 1 mM), manganese (0.1 and 1 mM) and strontium (0.1, 1 and 10 mM).

II. *The relaxing effect of metal ions on glycerol-extracted muscle fibres.*

As already shown some metal ions in adequate concentrations inhibit the isometric contraction. Therefore, it would seem of interest to determine if these metal ions are also able to relax fibre bundles that have been previously contracted by ATP. The effects were studied in the absence of ATP and in the presence

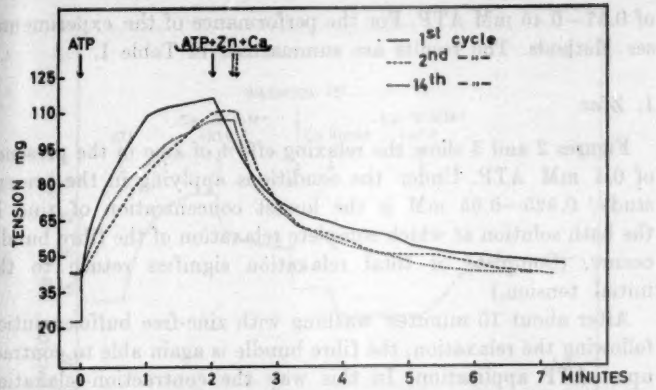


Fig. 2. Repeated contraction-relaxation cycles produced in the same fibre bundle by 0.1 mM zinc and 10 mM calcium in the presence of 0.37 mM ATP. Ordinate: Tension in mg. Abscissa: Time in min. after the start of the respective contraction cycle. The initial tension of the bundle: 22.2 mg. Contraction induced by 0.37 mM ATP. Relaxation brought about by 0.1 mM $ZnCl_2$ + 10 mM $CaCl_2$ + 0.37 mM ATP. Between the different cycles the fibre bundle is washed with buffer solution for 30 min. Cross section of the fibre bundle: 140 × 257 μ.

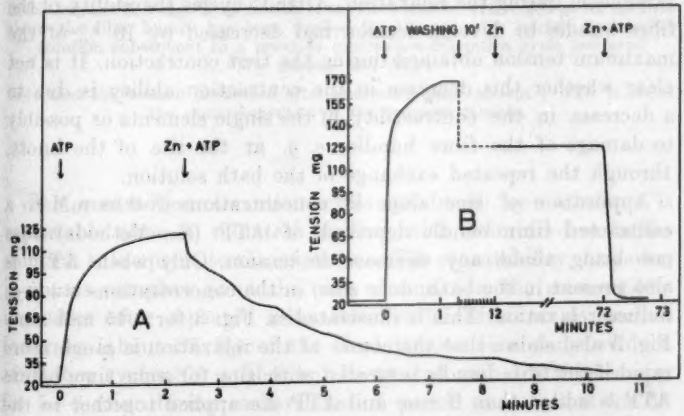


Fig. 3. Effect of zinc on a contracted fibre bundle in presence and absence of ATP. Ordinate: tension in mg. Abscissa: time in min. Initial tension of the bundle: 22.2 mg. Contraction induced by 0.40 mM ATP.

A. Relaxation produced in a contracted fibre bundle by 0.05 mM $ZnCl_2$ and 0.40 mM ATP without pretreatment with zinc. Cross section of the fibre bundle: 108 × 182 μ.

B. After contraction the fibre bundle is washed with buffer solution for 10 min. and immersed in 0.05 mM $ZnCl_2$ alone for 60 min; the zinc alone causes no relaxation. The solution is then exchanged for 0.05 mM $ZnCl_2$ + 0.40 mM ATP, which relaxes the bundle. Cross section of the fibre bundle: 108 × 206 μ.

Note the rapid relaxation by zinc and ATP after pretreatment of the fibre bundle with zinc as compared with the slow relaxation in Fig. 3 A.

of 0.37—0.40 mM ATP. For the performance of the experiments, see Methods. The results are summarized in Table I.

1. *Zinc.*

Figures 2 and 3 show the relaxing effect of zinc in the presence of 0.4 mM ATP. Under the conditions applying in the present study, 0.025—0.05 mM is the lowest concentration of zinc in the bath solution at which complete relaxation of the fibre bundle occurs. (Complete or total relaxation signifies return to the initial tension.)

After about 15 minutes' washing with zinc-free buffer solution following the relaxation, the fibre bundle is again able to contract upon ATP application. In this way the contraction-relaxation cycle may be repeated several times. In one experiment (Fig. 2) 14 cycles were performed in the same fibre bundle. After each relaxation the bundle was washed for 30 minutes with zinc-free buffer solution. No decrease in the ability to relax could be established in this experiment; the tension always returned to the same level during the relaxation. After 13 cycles the ability of the fibre bundle to develop tension had decreased by 10 % of the maximum tension obtained during the first contraction. It is not clear whether this decrease in the contraction ability is due to a decrease in the contractility of the single elements or possibly to damage of the fibre bundle, *e. g.* at the site of the knots, through the repeated exchange of the bath solution.

Application of zinc alone in concentrations ≤ 0.88 mM to a contracted fibre bundle deprived of ATP (see Methods) does not bring about any decrease in tension. Only when ATP is also present in the bath, does zinc, in the concentrations studied, induce relaxation. This is illustrated in Fig. 3 for 0.05 mM zinc. Fig. 3 also shows that the course of the relaxation is much more rapid if the fibre bundle is treated with zinc for some time before ATP is added than if zinc and ATP are applied together to the contracted fibre bundle.

2. *Cadmium.*

In the presence of ATP, cadmium produces relaxation of a contracted fibre bundle similarly to zinc. However, in order to obtain complete relaxation by cadmium it is necessary to use a more than 4 times higher concentration.

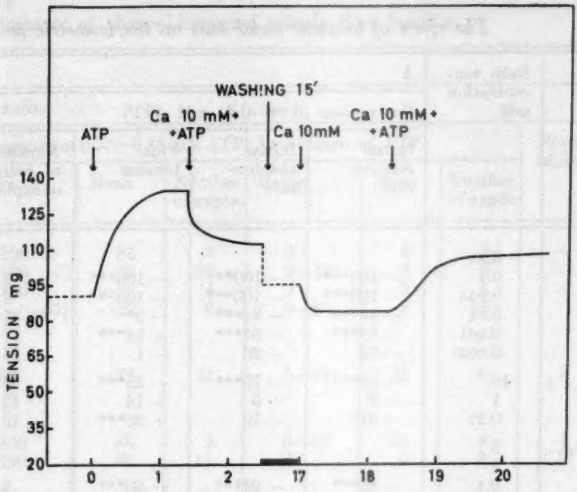


Fig. 4. Effects of 10 mM calcium on a contracted fibre bundle in presence and absence of ATP. Ordinate: Tension in mg. Abscissa: Time in min. Initial tension of the bundle: 22.2 mg. Cross section of the fibre bundle: 112 × 280 μ. At the first arrow the fibre bundle has been washed for 15 min. with calcium-free buffer solution subsequent to a previous contraction-relaxation cycle produced by calcium and ATP.

Note that the tension developed after immersion into calcium + ATP is about the same regardless of the previous tension.

The contraction-relaxation cycle produced by cadmium is repeatable after 30 minutes' washing with cadmium-free buffer solution subsequent to the relaxation. The repeatability, however, is not as complete as in the case of zinc, *i. e.* the contractility decreases after repeated relaxations with cadmium. A marked lowering (about 25 %) of the final tension is already evident after four cycles (for a fibre bundle treated with 1 mM cadmium for 5—6 min. during each relaxation period and washed for 30 min. with cadmium-free buffer solution after the relaxation).

3. *Mercury.*

A slow relaxation is obtained with 0.1 mM mercury in the presence of ATP. About 15 min. is usually required to attain total relaxation. When mercury and ATP are applied together to a contracted fibre bundle that has lost some of its tension through

Table

The effect of bivalent metal ions on the isometric tension

Metal	Bath concentration mM	A			
		Contraction (0.22—0.24 mM ATP)			
		Initial relative rate	Initial absolute rate	Final tension	Number of pairs of expts.
$ZnCl_2$ ...	0.88				
	0.1	—100***	—100***	—100***	4
	0.025	—100***	—100***	—100***	4
	0.01	—81***	—82***	—9*	17
	0.001	—68***	—64***	+14***	17
	0.0001	—24	—26	—1	14
$CaCl_2$ ...	10	—68***	—75***	—23***	14
	1	—8	+6	+14	12
	0.32	—31*	—16	+26***	12
$CdSO_4$ ..	5				
	1				
	0.1	—82***	—94***	—60***	4
	0.01	+1	+29	+21*	7
$HgCl_2$...	0.1	—100***	—100***	—100***	2
	0.01	—100***	—100***	—100***	3
	0.001	—65	—61	+27	4
	0.0001	—18	—7	+16	5
$MgCl_2$...	10	+94***	+65	—17	8
	0.2	—40	—43**	—10	12
$CoCl_2$ ...	10				
	1	+5	—21	—27*	7
	0.1	+43	+64**	+12	4
$MnCl_2$...	10				
	1	—12	—14	—5	7
	0.1	+11	+11	+1	4
$SrCl_2$ ...	10	—41	—42	—6	8
	1	—37	—36	+1	5
	0.1	—26	—17	+5	5

Part A: The effect on the contraction given as the difference between the mean of the tests and the mean of the controls in percentage of the controls. The degree of significance has been calculated using Fisher's t-test; *** = $P < 0.001$, ** = $0.001 < P < 0.01$, * = $0.01 < P < 0.05$.

removal of ATP (see Methods), there is an initial rise in tension followed by a slow relaxation. This course is probably due to the slowness of the accumulation of mercury in the fibre bundle.

In the absence of ATP, 0.1 mM mercury brings about a lowering of the tension in addition to the decrease in tension that follows the removal of ATP from a contracted fibre bundle.

1.

development of glycerol-extracted muscle fibre bundles.

B						
Relaxation						
In presence of 0.37—0.40 mM ATP			In absence of ATP			Reversibility
Range	Mean	Number of expts.	Range	Mean	Number of expts.	
49—100	82	5	0	0	3	+
75—100	93	20	0—10	2	6	+
93—100	96	4	0	0	4	+
—16—(—57)	—27	6	0—8	1	10	
16—30	22	12	5—78	45	6	+
54—100	86	9	0—52	18	4	+
71—100	91	11	0	0	2	+
41—100	86	17	50—80	64	5	0
27—42	35	3	0—23	12	4	+
6—14	10	2				
14—49	26	3	0	0	1	+
9—26	17	2				
5—(—21)	—6	8	6—53	32	7	+

Part B: The relaxing effect on the fibre bundles previously contracted by 0.37—0.40 mM ATP. The relaxation, the drop in tension, is expressed in percentage of the preceding tension obtained in the one series in the presence of 0.37—0.40 mM ATP and in the other series after removal of ATP by washing. Negative values refer to increase in tension. Reversibility: + reversible, 0 irreversible.

After 15 minutes' treatment of the bundle with 0.1 mM mercury the lost contractility cannot be restored by washing with mercury-free buffer solution. The washing has been performed with 40 ml solution for time intervals ranging from 30 min. to 3 hrs.

4. *Calcium and strontium.*

Both calcium and strontium in 10 mM concentration are able to produce relaxation of a contracted fibre bundle in the absence of ATP. In the presence of 0.4 mM ATP, a smaller relaxation (see Table I) is obtained with 10 mM calcium, while 10 mM strontium is without relaxing effect. Fig. 4 illustrates typical effects of calcium.

The contraction-relaxation cycle produced by calcium and strontium is repeatable; the contractility is restored after 5—10 minutes' washing with buffer solution subsequent to the relaxation.

5. *Manganese and cobalt.*

A slight relaxation is obtained with cobalt and manganese (1 mM and 10 mM) in the presence of 0.4 mM ATP.

Discussion.

At relatively high concentrations the metals zinc, calcium, cadmium and mercury inhibit the entire course of the contraction. This in itself is not a surprising effect. At lower concentrations, however, zinc and calcium significantly increase the final tension, while they inhibit the initial development of tension (zinc) or have no significant effect on the initial phase (calcium).

Thus, there is a qualitatively different action on the final tension at high and low concentrations of zinc and calcium. However, both effects may be explained by a simultaneous decrease in tension of the single element and an inhibition of the ATPase activity. Taking the tension of the bundle as a whole, the decrease in tension developed by the single element will then be compensated to a certain degree or even overcompensated by activation of deeper layers of the fibre bundle made possible by inhibition of the ATPase activity and consequent deeper penetration of ATP. The effect through activation of deeper layers will be negligible in the most initial phase of the contraction but will grow increasingly significant during the continued course up to the final tension. This may explain the differences in the effect of the individual metals at low concentrations (zinc 0.001 mM and calcium 0.32 mM) on the initial phase and on the final tension.

The effect of metals on the initial phase of the contraction may

further be complicated by the inconstancy of the free metal ion concentration within the fibres during the contraction. Before the addition of ATP the fibres contain the metal in a certain concentration, which is in equilibrium with the metal concentration of the bath. With the addition of ATP, however, the concentration of free metal ions in the bath solution decreases, owing to formation of an ATP-metal complex (MARTELL and SCHWARZENBACH 1956, RAAFLAUB 1956, WALAAS 1957, G. WEIZEL, personal communication concerning zinc, calcium and magnesium) and the equilibrium between the free metal ion in the bath and the protein-bound metal in the fibres is changed. This will cause a change in protein-bound metal unless the protein is completely saturated even at the lower concentration of free ions. The distribution of free metal ions within the fibres is further complicated by the fact that ATP has only a certain, limited depth of diffusion into a fibre and a fibre bundle. Moreover, ADP and AMP are also able to form metal complexes although in a lesser degree than ATP (MARTELL and SCHWARZENBACH 1956, RAAFLAUB 1956, WALAAS 1957). In consequence, the distribution of free metal ions within the fibres will not be uniform even at steady state. The complications caused by the change in amount of protein-bound metal occurring during the development of the contraction has not been considered in earlier studies of metal ion effects in these systems.

The final tension is insignificantly lower with 10 mM and 0.2 mM than with 1 mM magnesium. This agrees with observations by RANNEY (1955) working with thick fibre bundles from dog heart trabecular muscle. He found an optimum at 3 mM magnesium. KOREY (1950) working with thick rabbit psoas fibre bundles found 10 mM magnesium to be optimal.

Cobalt and manganese can compensate for the decrease in function that arises in the actomyosin thread (GERENDAS 1941, BOWEN 1952) or the glycerol-extracted muscle fibre (WATANABE and SLEATOR 1957: manganese) when the magnesium concentration is reduced. According to BOWEN (1952), the stimulating effect on the shortening of actomyosin threads is equally great for magnesium, cobalt and manganese when these metals are used in equimolar concentrations. This does not prove, however, that the mechanism of action is the same for the three metal ions or that magnesium can be fully replaced by cobalt and manganese. In fact, according to the foregoing discussion, a comparison of

the total metal concentrations in the bath does not necessarily give a decisive answer to the question whether or not a metal can be replaced atom for atom by another metal at the strategic sites of the contractile protein or of the substrate. Furthermore, a strictly quantitative comparison of the effects of different metals on whole fibres or fibre bundles is difficult because of the aforementioned fact that a change in the ATPase activity modifies the depth of diffusion of ATP into the fibres.

HASSELBACH (1953) has shown that zinc in a comparatively high concentration, >4 mM, reversibly inhibits the tension development of glycerol-extracted muscle fibres and fibre bundles.[1] As shown in the present work, the inhibiting effect of zinc is quite evident even at 0.01 mM concentration in the bath solution. At 0.025 mM zinc no contraction can be induced by 0.23 mM ATP.

According to HASSELBACH (1953) and HASSELBACH and A. WEBER (1955) mersalyl is the only one of the ions and compounds of heavy metals studied [Ag^{+}, Fe^{++}, Fe^{+++}, Hg^{++}, Cu^{++}, Zn^{++}, $KMnO_4$, K_3FeCN_6, Cu-glycine, Salyrgan® (mersalyl), Oxarsan® (m-amino-p-hydroxyphenyl arsinous oxide) and p-chloromercuribenzoic acid] which produces relaxation of contracted fibres and fibre bundles in the presence of ATP.[1] As demonstrated in the present work, however, relaxation can be produced by several bivalent metal ions. The discrepancy probably depends upon differences in the concentrations of the various components used in our systems.

Among the relaxation-inducing metal ions studied, zinc seems to be of particular interest. Zinc is effectively relaxing even at a bath concentration as low as 0.025—0.05 mM in the presence of 1 mM magnesium and 0.4 mM ATP. Furthermore, the contraction-relaxation cycle is repeatable many times. The physiologic content of zinc in skeletal muscle is comparatively high, *i. e.* according to KOGA (1934) 0.5 mmole, HAMM (1955) 0.6 mmole, KOCH *et al.* (1956) 1.1 mmole, EDMAN (unpublished) 0.8 mmole; all values represent content per kilogram wet tissue.

As shown in this work, mercury in rather low bath concentrations relaxes a contracted fibre. On the other hand, LAKI and BOWEN (1955) reported that HgI_2 (150—500 mM) + KI (300—1 000 mM) caused shortening and tension development in glycerol-

[1] According to a personal communication from Dr. HASSELBACH the bath solution was unbuffered having a pH of 7.0 and containing 80 mM KCl and 5 mM magnesium. The ATP concentration was 5 mM.

extracted rabbit psoas bundles and myosin-B threads. These findings, however, are not completely comparable with the results presented in the present study since the mercury concentrations in the experiments of LAKI and BOWEN were very much higher than those used in this work.

It has been proposed (WEBER and PORTZEHL 1952, 1954) that the relaxing effect of the organic mercury compound mersalyl is due to its inhibition of the ATPase activity, thereby allowing ATP to act only as a "plasticizer". A similar mechanism of action is also possible for the relaxing effects obtained in the present work by zinc, cadmium and mercury. However, in the case of mercury, it is improbable that the action is due only to an inhibition of the ATPase activity, since mercury is also able to relax a fibre bundle in the absence of ATP. This suggests a relaxing action of mercury itself by its coupling to the protein. An interesting finding in this connection is the ability of mercury in the absence of ATP to produce a viscosity drop in actomyosin solutions (KUSCHINSKY and TURBA 1951, TURBA and KUSCHINSKY 1952: mersalyl) and to inhibit the combination between actin and myosin (BAILEY and PERRY 1947: p-chloromercuribenzoate, KUSCHINSKY and TURBA 1951, and TURBA and KUSCHINSKY 1952: mersalyl). In view of the high affinity of mercury for SH-groups, blocking of these groups has been held responsible for the effect of mercury compounds on the contractile system. This assumption may be correct. However, at least in the case of zinc and cadmium it is still uncertain if coupling to SH-groups is of *functional* relevance considering the affinity of these metals, albeit much smaller, for other groups in the protein as well, *e.g.* the imidazole groups (KLOTZ 1953, GURD and WILCOX 1956).

A more detailed study of the effects of zinc on glycerol-extracted fibres will be published separately.

Summary.

1. The effect of several bivalent metal ions on the ATP-induced contraction of glycerol-extracted rabbit psoas fibre bundles has been studied under highly isometric conditions. The relaxing effect of these metals on previously contracted fibre bundles in the presence of 1 mM magnesium and in the presence or absence of ATP has also been investigated.

2. Zinc ($\geq$ 0.01 mM), cadmium ($\geq$ 0.1 mM), calcium ($\geq$ 10 mM) and mercury ($\geq$ 0.01 mM) partly or wholly inhibit the contraction. In lower concentrations, however, zinc (0.001 mM) and calcium (0.32 mM) produce an increase in the final tension but have no significant effect (calcium) or an inhibiting action (zinc) on the initial phase of the contraction.

3. In the presence of 0.4 mM ATP, 0.025—0.88 mM zinc induces relaxation of a previously contracted fibre bundle. The contraction-relaxation cycle can be repeated many times. In the absence of ATP, no relaxing effect could be demonstrated for zinc ($\leq$ 0.88 mM).

4. A less reversible relaxation, as compared with that of zinc, is obtained with cadmium (5 mM and 1 mM) in the presence of 0.4 mM ATP. Mercury (0.1 mM) produces a slow relaxation together with 0.4 mM ATP; the relaxation is irreversible after 15 minutes' incubation with mercury, however. Calcium, cobalt and manganese (10 mM) bring about a slight decrease in tension of a contracted fibre bundle in the presence of 0.4 mM ATP.

5. A certain decrease in tension can be demonstrated for calcium (10 mM), strontium (10 mM) and mercury (0.1 mM) in the absence of ATP.

6. In the discussion it is stressed that the complexing of metal ions by ATP greatly complicates the interpretation of findings obtained in studies of the interactions of metals, ATP and contractile proteins.

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The Release of Catechol Amines from the Amine Containing Granules of the Adrenal Medulla.

By

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Isolated amine granules from the adrenal medulla show no or only a very slow release of adrenaline and noradrenaline when kept at 0° and at about neutral pH. The amines are readily released, however, if the temperature is elevated or the pH decreased below 6 (HILLARP, LAGERSTEDT and NILSON 1953, HILLARP and NILSON 1954, EADE 1957). Particularly the "spontaneous" release at elevated temperature has been further studied in the present paper as this process may have some bearing on the problem of the amine liberation in vivo upon indirect stimulation of the adrenal medulla.

Material and Methods.

Isolation of the medullary granules: The specific granules of cow adrenal medulla were isolated in 0.3 M sucrose by differential centrifugation as described in a previous paper (HILLARP 1958 c). The most easily sedimentable granules were washed twice and finally suspended in a small volume of 0.3 M sucrose.

The following abbreviations are used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5-phosphate; P_o, inorganic P; P_a, acid-labile P.

Incubation at elevated temperature: The granules were incubated with or without shaking at +20° to +38° in 0.3 M sucrose or various electrolytic media usually containing NaCl or KCl (final concentration, 0.05 to 0.18 M) and histidine-HCl (pH 7.5; final concentration, 0.04 M). The media were made iso-osmotic or slightly hyperosmotic to 0.3 M sucrose by adding sucrose or KCl. Aliquots were taken at various time intervals, cooled to 0° C and centrifuged for 30 minutes at 38,000 × *g*. The clear supernatant was precipitated with trichloroacetic acid and the granule sediment extracted with 0.4 N perchloric acid. Catechol amines, protein-N, P_o, P_s and adenosinephosphates were determined as described previously (HILLARP 1958 b). The total amount of soluble proteins in the granules was determined from an aliquot of the original granule suspension by lysis in 0.015 M KCl (cf. HILLARP 1958 c).

When the release of ATP from the amine granules was studied, the incubations were carried out at +37° in a mixture containing 0.05 M sucrose, 0.05 M KCl, 0.04 M histidine-HCl (pH 7.5), 0.005 M $MgCl_2$, 0.005 M NaF, 0.075 M glucose and 1.0 mg of hexokinase per ml. Control experiments showed that the granule suspensions exhibited a glucose-6-phosphatase activity but that this activity was completely inhibited by the addition of NaF. The NaF concentration used did not diminish the ATP trapping efficiency of the hexokinase-glucose system. The hexokinase (the type II preparation from yeast of the Sigma Chemical Company) was stored at —25° as a stock solution in 0.75 M glucose. When incubated together with ATP (0.005 M; pH 7.5) in the mixture described above no inorganic P was liberated.

All solutions were made in glass distilled or deionized water.

Incubation at lowered pH: The pH of granule suspensions in 0.3 M sucrose was adjusted to about 5 by addition of small amounts of a M acetic acid-acetate buffer (pH 4.5). The suspensions were kept at +4° for one to four hours and then the pH was adjusted to about 6.5 by addition of a small volume of a M $NaHCO_3$ solution. This is necessary in order to solubilize any proteins that may have been released from the granules. The suspensions were centrifuged as described above and catechol amines and proteins were determined in the supernatant and granule sediment.

Determination of lactic acid: Cow adrenal medulla was extracted with 0.4 N perchloric acid. The extracts were neutralized with KOH and passed through a cation exchanger (Dowex 50; pH 6) to remove the catechol amines. Lactic acid was thereafter determined according to the method of BARKER and SUMMERSON (1941) as modified by STONE (1948).

Results.

I. *The "spontaneous" amine release at elevated temperatures.*

No or a very slow release of catechol amines occurs when isolated medullary granules are kept at 0° to +4° C in sucrose, NaCl or KCl media iso-osmotic (or hyperosmotic) to 0.3 M sucrose.

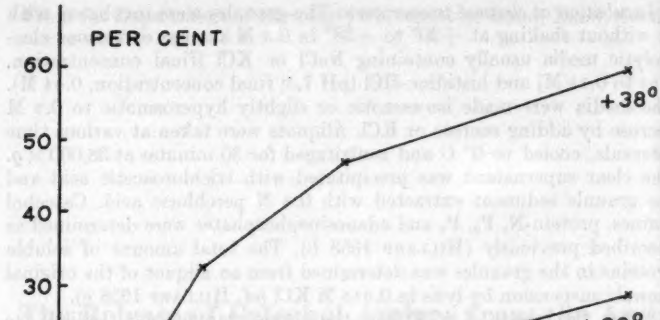

Fig. 1. Release of catechol amines from granules incubated in a medium containing 0.05 M sucrose, 0.14 M KCl and 0.02 M histidine-HCl (pH 7.5).

If the temperature is elevated, a release takes place, however. At +20° the amines are only slowly liberated but the release is greatly accelerated by raising the temperature (Fig. 1).

In several experiments the time course of the release was studied during the first half hour. No initial lag phase was observed. When granules were preincubated at +37° for 30 minutes and then cooled to 0°, the amine liberation was greatly reduced.

The amine liberation is not appreciably altered by the addition of buffers, phosphate, NaF (0.04 M), Ca^{++}, Mg^{++} and KCN (0.001 M) or by replacing O_2 by N_2. No inhibition has been obtained with AMP, ADP or ATP (0.005 M). As several metal ions (Zn^{++}, Cd^{++}, Cu^{++}, Mn^{++}, Al^{+++}, Fe^{+++}) give a highly accelerated amine release at low concentrations (< 0.001 M) (HILLARP 1958 b), it seemed possible that the "spontaneous" release was caused by metallic impurities. However, neither versene (0.01 M) nor various thiol compounds (glutathione, cysteine, cystine, BAL; 0.001 M) inhibited the amine liberation at elevated temperature.

The amine release in vivo does not seem to be due to a destruc-

tion of the medullary granules (CARLSSON and HILLARP 1956, 1957, SCHÜMANN 1958). It is therefore of considerable interest to know whether the amines during the "spontaneous" release in vitro are liberated merely as a result of a decomposition or destruction of the granules.

If the granules are damaged by various means, for example, mechanically or by osmotic lysis (cf. HILLARP and NILSON 1954), large amounts of soluble proteins are released together with the catechol amines. No or only small amounts of the soluble proteins in the granules are liberated, however, even if about half of the amines leave the granules during incubation at +37° (Tables I to III). This strongly speaks against the view that the granules are merely decomposed at elevated temperatures. — Control experiments have shown that the soluble granule proteins, if released by lysis before the incubations, do not precipitate during the incubations.

The catechol amines are stored in the granules together with about an equivalent amount of adenosinephosphates (mainly ATP) and the ATP is released or broken down during the amine secretion in vivo (HILLARP, HÖGBERG and NILSON 1955, FALCK, HILLARP and HÖGBERG 1956, CARLSSON and HILLARP 1956, 1957, HILLARP 1958, SCHÜMANN 1958). Correspondingly, the ATP in the granules decreases parallelly with the amines when isolated granules are incubated at elevated temperatures (Tables I and III). The ADP and AMP, however, do not drop in the same proportion. The total amount of AMP may even increase. A relatively large part of the AMP found may not belong to the granules, however, but may come from the AMP in the suspension medium where large amounts accumulate owing to the ATP break-down (see below).

No or only small amounts of ATP appear in the medium during the incubations. There is instead an accumulation of AMP and inorganic P. The ATP therefore is either split before the release from the granules or dephosphorylated after having left them. The amine granules have a highly active ATPase, readily attacking any ATP added to the suspension medium, which is probably bound to the granule membrane. The suspensions also exhibit an adenylate kinase activity (HILLARP 1958 a). On account of the presence of an ATPase it is tempting to speculate on the possibility that the amine release is brought forth by an ATP break-down. This has been tested in two ways.

Table I.

Release of catechol amines and changes of adenosinephosphates in granules incubated at +37°. Incubation media: A, 0.24 M sucrose — 0.02 M KCl — 0.04 M histidine-HCl pH 7.5; B, 0.06 M sucrose — 0.14 M KCl — 0.04 M histidine; C, 0.12 M sucrose — 0.05 M KCl — 0.04 M histidine — 0.005 M $MgCl_2$ — 0.005 M NaF — 0.075 M glucose; D, as C but without NaF.

Exp.	Incubation at + 37° Minutes	Catechol amines		ATP		ADP	AMP	Soluble proteins
		Total μmoles	Release Per cent	Total μmoles	Decrease Per cent	Total μmoles	Total μmoles	Release Per cent
A	0	50.5		10.8		1.05	0.48	
	20	44.5	11.9	9.45	12.5	0.98	0.47	4.1
	40	41.9	17.0	8.95	17.1	1.10	0.68	2.8
	80	38.3	24.2	8.00	25.7	1.10	0.67	5.0
	160	32.5	35.7	7.35	32.0	0.95	0.57	5.5
B	0	47.5		9.90		1.15	0.68	
	40	38.5	18.9	8.05	18.7	1.10	0.58	3.8
	80	34.2	28.0	7.20	27.3	1.20	0.65	4.9
	160	24.4	48.6	5.05	49.0	0.85	0.56	1.2
C	0	36.3		7.60		0.73	0.35	
	40	32.2	11.3	6.85	9.9	0.72	0.32	3.8
	80	28.0	22.9	6.15	19.1	0.64	0.33	1.9
	160	21.6	40.5	4.70	38.2	0.61	0.29	3.8
D	0	92.0		19.3		2.65	1.40	
	120	50.4	45.2	10.5	45.6	2.20	1.55	2.1

Table II.

Release of catechol amines and inhibition of the granule ATPase by ADP. The granules were incubated in a medium containing 0.075 M sucrose and 0.12 to 0.14 M KCl with or without ADP (0.0125 M) and ATP (0.0025 M).

Incubation at + 37°	Release of catechol amines Per cent	Increase of P_e in the medium µg	Release of soluble proteins Per cent
Control 60 Min.	31.2	221	1.2
ADP 60 Min.	35.0	96	1.9
ATP 60 Min.	37.6	452	3.4
ADP+ATP 60 Min. ...	36.3	165	2.5
Control 80 Min.	43.7	136	2.7
ADP 80 Min.	40.5	59	2.1
ATP 80 Min.	45.7	306	4.3
ADP+ATP 80 Min. ...	45.2	120	3.9

If the ATPase is linked to the mechanism for the amine release, it may be supposed that ATPase inhibitors slow down the release. Unfortunately, the usual inhibitors (such as p-chloromercuribenzoate and salyrgan) cause a lysis of the granules (cf. D'Iorio 1957, Hillarp 1958 a). ADP, however, inhibits the ATPase of the medullary granules as in the case of myosin (Kalckar 1944) and of mitochondria (Kielley and Kielley 1953, Potter, Siekevitz and Simonson 1953). When a high concentration of ADP is present in the suspension medium, the liberation of inorganic P from added ATP is considerably reduced (Table II). This is not accompanied by a lysis of the granules, as shown by the absence of protein release. ADP also inhibits the dephosphorylation of the ATP that disappears from the granules during incubations at elevated temperature. This is seen from the fact that considerably smaller amounts of inorganic P appear in the medium (Table II). In spite of this the "spontaneous" amine release is not decreased.

A more direct approach to the problem is to introduce an ATP trapping system in the suspension medium. Some typical experiments with hexokinase-glucose are recorded in Table III. The decrease of the granule ATP runs parallel with the amine release but only 10 to 20 per cent of the labile phosphate of the ATP that disappeared was recovered as inorganic P. Furthermore, only a small part of the P_e that disappeared from the granules was found as P_e in the medium. In contrast to this, if hexokinase was omitted, the larger part was recovered as inorganic P and the remaining

Table III.

Changes of P_0 and P_2 in granules and medium during incubations with or without hexokinase-glucose. Incubation medium as in Table I: C.

Incubation at + 37° Minutes	Hexokinase	Granules Per cent decrease		P_0 μmoles			P_2 μmoles			Soluble proteins Per cent release
		Amines	ATP	Granules	Medium	Changes	Granules	Medium	Changes	
0	+			2.58	1.94		32.9	0.84		
120	+	48.2	45.0	1.42	4.23	+ 1.13	19.4	3.90	—10.4	2.0
120	—	44.7	44.1	2.05	14.3	+11.8	19.1	3.75	—10.9	1.5
0	+			1.60	0.71		15.9	0.13		
120	+	53.8	53.8	1.22	2.65	+1.56	8.00	0.55	—7.50	3.3
120	—	47.4	49.5	1.41	9.20	+8.30	7.85	0.62	—7.55	1.1
0	+			2.58	1.10		25.9	0.14		
120	+	41.0	40.5	1.60	3.50	+1.42	15.9	0.94	—9.20	
0	+			3.00	3.10		23.4	0.23		
120	+	46.5	46.8	1.81	5.80	+1.51	13.5	1.11	—9.00	

part as P_s.[1] This clearly shows that more than half of the ATP which disappeared from the granules must have left the granules without having been dephosphorylated.

Both the hexokinase and the ATPase inhibition experiments speak against the view that the "spontaneous" amine release is brought forth by an ATP break-down. Instead it seems highly probable that ATP is liberated along with the amines and then secondarily split into AMP and inorganic P by the combined activity of the ATPase and adenylate kinase of the medullary granules.

It is difficult to decide whether the "spontaneous" amine release is caused by a damage to the granules during the isolation procedure. The very high stability of the granules at 0° to +4° does not favor this view. Moreover, the medullary granules in homogenates of adrenal medullas from cats show the same release even if the preparations are made at 0° immediately after killing the animals.

II. *The amine release at decreased pH.*

Isolated medullary granules release their amines even at 0° to +4° if the pH of the medium is lower than 6. It is therefore possible, although not very probable, that the amine liberation in vivo is due to a pH decrease in the cells brought about, for instance, by glycolysis.

From this point of view, it is of interest that the adrenal glands some time after death show large amounts of lactic acid (KENDALL 1932). Cow adrenal medulla obtained from glands removed about 30 minutes after death has been found to contain between 2 and 3 mg lactic acid per gram tissue (wet weight). Considerable quantities of acid may thus be produced in the cells by glycolysis. In spite of this the pH of the medulla (press juice) does not lie below 6.2. However, it is quite possible that pH values below 6 may be obtained in vivo at least temporarily.

There is suggestive evidence that the intragranular proteins play a role in the storage of the catechol amines (HILLARP 1958 b). Their isoelectric pH is 4.2 and they begin to flocculate at about pH 5.5 (HÖGBERG and HILLARP, unpublished observations). The change in their charge and solubility may thus be a possible explana-

[1] The presence of P_s in the medium may be explained on the basis of an inhibition of the ATPase and adenylate kinase by NaF (cf. HILLARP 1958 a).

tion for the amine release at decreased pH. However, it has been repeatedly found that isolated granules are rapidly severely damaged at a pH of about 5. At this pH 30 to 80 per cent of the amines are liberated during one to four hours (+4°), but at the same time almost as large a part of the soluble proteins are released from the granules. This speaks against the theory that the amine release in vivo is due to a decreased pH in the medullary cells.

Discussion.

Several observations show that the amine granules of the adrenal medulla are not true secretory granules — that is, they do not leave the cells during secretion (HILLARP, HÖKFELT and NILSON 1954, CARLSSON and HILLARP 1956, 1957, WETZSTEIN 1957, SCHÜMANN 1958). Consequently the stored catechol amines and ATP must be released from the granules during this process.

It is difficult to decide whether the "spontaneous" release in vitro is a phenomenon related to the physiologically occurring secretion. The granules — like mitochondria (see *e. g.* KIELLEY and KIELLEY 1951, POTTER, SIEKEVITZ and SIMONSON 1953, SIEKEVITZ and POTTER 1955) — may be damaged when incubated in an abnormal milieu at elevated temperatures. The non-existence of a protein release and of an initial lag phase in the amine liberation and the fact that a preincubation of the granules at +37° does not give irreversible changes leading to a continued release, speak against this view, however.

The processes in the cells that finally lead to an amine release are as yet unknown. It has been found, however, that anoxia of the denervated medulla (O_2-deprivation; KCN) gives a prompt discharged of catechol amines (ZWEMER and NEWTON 1928, BÜLBRING, BURN and DE ELIO 1948, COMLINE and SILVER 1958). It may thus be that the liberation is due to an inhibition of oxidative processes in the cells that keep a "spontaneously" operating release in check. If this is the case, it is easily understood that isolated granules liberate their amines under conditions not permitting the oxidative processes normally occurring in the living cell.

The possibility that the highly active ATPase localized in the medullary granules plays an active role in the release mechanism has been considered in previous investigations (cf. HILLARP 1958 a). This is not supported by the observations in the present study.

During the "spontaneous" amine liberation at least a large part of the stored ATP leaves the granules without being dephosphorylated. It may thus not seem so unlikely as previously thought that ATP may be secreted from the medulla along with the sympathomimetic amines. This is at present being studied in perfusion experiments.

Summary.

1. No or a very slow release of catechol amines occurs when the granules isolated from cow adrenal medulla are kept at 0° to +4° C. If the temperature is elevated, a liberation takes place both in sucrose and electrolytic media. No inhibitors for this "spontaneous" release have been found.

2. The ATP stored in the granules decreases parallelly with the catechol amines. The ADP and AMP, however, do not drop in the same proportion. No or only small amounts of the soluble proteins in the granules are liberated during the amine release.

3. The experiments speak against the view that the "spontaneous" amine release is brought forth by an ATP break-down. Instead it seems highly probable that ATP is liberated along with the amines and then secondarily split into AMP and inorganic P by the combined activity of the ATPase and adenylate kinase of the medullary granules.

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Intracellular Recording from Cells in Clarke's Column.

By

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The technique of intracellular recording (BROCK, COOMBS and ECCLES 1952) has been extensively applied in investigations on motoneurones and has led to a considerable advance in our knowledge regarding their properties (COOMBS, ECCLES and FATT 1955 a, FRANK and FUORTES 1956), the mechanism of synaptic actions (COOMBS, ECCLES and FATT 1955 b, c), the initiation of impulses (ARAKI, OTANI and FURUKAWA 1953, ARAKI and OTANI 1955, FATT 1957 a, b, FUORTES, FRANK and BECKER 1957, COOMBS, CURTIS and ECCLES 1957 a, b) and the pattern of connections of the cells (ECCLES, FATT and LANDGREN 1956, ECCLES, ECCLES and LUNDBERG 1957 a, b).

The question may be raised whether the properties of motoneurones as disclosed by these investigations are representative of other types of cells in the central nervous system. For this purpose we have attempted to record intracellularly from Clarke's column cells, which are the cells of origin of the dorsal spino-cerebellar tract with its direct connections to the anterior lobe of the cerebellum. The physiology of this tract has been intensively investigated through intra-axonal recording from fibres in Flechsig's fasciculus

(LAPORTE, LUNDBERG and OSCARSSON 1956 a, b, LAPORTE and LUNDBERG 1956, LUNDBERG and OSCARSSON 1956, HOLMQVIST, LUNDBERG and OSCARSSON 1956). It contains two subgroups, one receiving monosynaptic excitation from muscle spindle afferents, the other from Golgi tendon organs. In both these subgroups the majority of neurones could be excited only from one muscle. Inhibitory actions with brief latency were also exerted by impulses in group I afferents. The major source of the inhibitory action was the antagonist muscle of the one supplying the excitatory, but some group I inhibition was supplied from other muscle nerves as well.

The present investigation has demonstrated that it is difficult to record intracellularly from Clarke's column cells. Nevertheless some findings of general interest have been made, which indicate that the mechanisms for synaptic excitation and inhibition and for the initiation of impulses are the same as in motoneurones.

Methods.

All experiments were performed upon cats under light pentobarbital sodium anaesthesia. The spinal cord was divided either in the mid-thoracic or upper lumbar region. The technique for intracellular recording has been described by BROCK *et al.* (1952). The microelectrodes, filled with 3 M KCl solution, were chosen to have resistances of 20—30 M-Ω and the grid current of the input cathode follower was less than 5×10^{-11} A. Both these factors tended to reduce damage to the impaled cells.

For antidromic activation of the cells Flechsig's fasciculus was dissected as described by LAPORTE *et al.* (1956 a) and mounted on stimulating electrodes. In some of the experiments the dissected fasciculus deteriorated due to oedema, and when this occurred antidromic identification of Clarke's column cells could not be secured. Nevertheless, these investigations have been included in the results because the location of the microelectrode tip in the cord in the third to fourth lumbar segments at 2 mm depth and close to the midline made it reasonably certain that the recording was made from the neurones of Clarke's column. Furthermore all the principal results reported in this paper have been found also on cells identified through antidromic invasion from Flechsig's fasciculus.

In order to reduce movements of the spinal cord the vertebral column was rigidly clamped to the frame supporting the micromanipulator (ECCLES *et al.* 1954). In some experiments a bilateral pneumothorax was made, the cat being artificially respired. Nevertheless, it was very difficult to record intracellularly from these cells; usually after a successful insertion there was a rapid decline of membrane potential with

a resulting increase in the frequency of the resting discharge. Furthermore, cells were difficult to locate owing to the virtual absence of extracellular potential fields, which can be correlated with the dense packing of the dendrites within the narrow confines of Clarke's column, and the consequent closed-field type of extracellular potential (cf. LORENTE DE NÓ 1947, 1953).

Results.

1. The antidromic and orthodromic activation.

The intracellular records in Fig. 1 A—D, show the orthodromic responses from a Clarke cell following stimulation of the nerve to quadriceps. In A, a submaximal group I volley evoked and excitatory postsynaptic potential (EPSP) of a size subliminal for activation. When the size of the incoming volley was increased in B and C the slope of the rising phase of the EPSP increased, showing that there was convergence from several primary afferent fibres onto this cell (SZENTÁGOTHAI and ALBERT 1955, LAPORTE *et al.* 1956 b, HOLMQVIST *et al.* 1956). The time course of the EPSP cannot be accurately determined because, when the records were being taken, the cell was discharging spontaneously at a frequency of about 50/sec (observe spontaneous spike at beginning of C). The after-potential following the spikes gave the sloping baselines that distorted most of the records. So far as can be determined the EPSP in A and F would last for about 10 msec, and a similar duration is also indicated in Fig. 2 A, but much shorter durations are seen in Fig. 5 A, C, D. Probably the EPSP in the intact Clarke cell has approximately the same duration as the monosynaptic EPSP of motoneurones (LLOYD 1946, BROCK *et al.* 1952), or in the cells of the ventral spino-cerebellar tract (OSCARSSON 1957), where by indirect or direct methods, values of about 15 msec have been found. In all of the records illustrated in this paper the resting membrane potential was below 50 mV. Consequently, we must expect that the time constant of the membrane was subnormal and this may be the reason for the briefer duration of EPSP's often found here, just as is observed with deteriorated motoneurones (cf. CURTIS and ECCLES 1958).

The antidromic invasion can be observed in Fig. 1 E to occur in two stages, as is particularly evident in the lower differentiated record. In normal motoneurones this two stage antidromic invasion is more prominent than in Fig. 1, but we have never failed to ob-

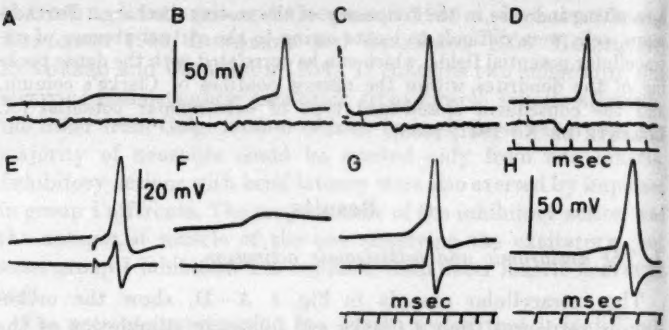

Fig. 1. The upper traces show intracellular records from a cell in Clarke's column, with the electrically differentiated records in the lower traces in E—G. The lower traces in A—D show the incoming volley, negativity being signalled by a downwards deflection. Stimulation of the quadriceps nerve in A—D and F—G. Record E shows the antidromic invasion evoked by stimulation of the dissected Flechsig's fasciculus 60 mm cranially to the site of micro-electrode recording in the third lumbar segment. Calibration for A—D shown between A and B and for E—G between E and F. Record H shows the orthodromic activation of another cell in Clarke's column, with a more marked degree of two-stage invasion particularly in the lower differentiated trace.

serve it in Clarke's cells despite the low membrane potential and the spontaneous background discharge.

On the other hand, Clarke's cells differ from motoneurones in that with orthodromic activation these two phases were not always discernible, although in Fig. 1 they are just visible in the differentiated record of G. Record H illustrates the most marked two stage invasion that we have observed. This cell had a spike potential of 70 mV and its resting discharge was as low as 5/sec, which approximates to the frequency usually observed in a normal cell as recorded in the axon, LAPORTE *et al.* (1956 c). The upper undifferentiated record shows that excitation was evoked by an EPSP of less than 10 mV, while the two stages of the invasion appear particularly well in the lower differentiated trace. By analogy from the findings on motoneurones, (ARAKI and OTANI 1955, FUORTES *et al.* 1957, COOMBS *et al.* 1957 a, b), these results suggest that in a Clarke's cell the soma has a higher threshold than the initial segment, and that on orthodromic activation excitation takes place in the initial segment.

On recording from axons in the dorsal spino-cerebellar tract it was observed that some units responded to a single presynaptic

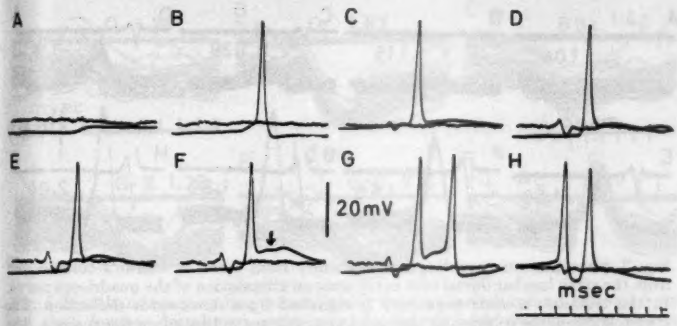

Fig. 2. Simultaneous recording, intracellularly from a cell in Clarke's column and from the sixth lumbar dorsal root entry zone. In the triphasic records negativity is signalled by a downwards deflection. A—G were obtained on stimulation of the nerve to posterior biceps-semitendinosus with increasing strengths of stimulation. In H the impulse evoked by the primary afferent volley was preceded by a spontaneously occurring impulse.

volley with a double spike (LAPORTE *et al.* 1956 b). Sometimes this occurred with a submaximal group I volley, but sometimes only when the group II fibres were included in the primary afferent volley. When the double spike discharge occurred with a submaximal group I volley, it was suggested that the second spike was due to the survival of transmitter action. On the other hand, when the second spike was evoked by impulses in group II fibres, its latency showed that they were acting monosynaptically. The synaptic processes underlying this double spike discharge are illustrated in Fig. 2. This cell received strong excitatory action from the combined nerve to posterior biceps and semitendinosus. Already the very weak volley in A evoked an EPSP, which increased with the slight increase of stimulus in B, causing excitation when the cell had been depolarized by 3 mV. With further increase of stimulus strength in C, D and E, the EPSP increased, as evidenced by the steeper initial rising phase, but now the spike potential was initiated so early that it arose from the EPSP with no separating inflection. However, it may be assumed that also in records C — G the spike was initiated at a level of depolarization that was not larger than the 3 mV in B. The spike potential in B and in the spontaneous first spike in record H was followed by the positive overshoot that is usually found in depolarized cells, being an effect of the increased potassium conductance during

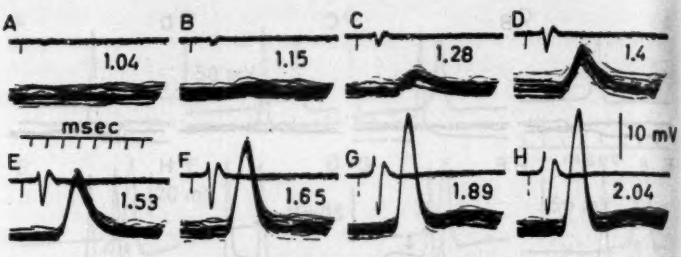

Fig. 3. Simultaneous recording intracellularly from a cell in Clarke's column and from the sixth lumbar dorsal root entry zone on stimulation of the quadriceps nerve. In the triphasic records negativity is signalled by a downwards deflection. The stimulus strengths relative to threshold strength are indicated on the records. The membrane potential was 30 mV when these graded spike-like potentials were recorded. A few minutes earlier at a membrane potential of 50 mV the usual all-or-none spike was obtained from this cell. About 40 traces are superimposed in each record.

and after the spike (cf. HODGKIN 1951, COOMBS, ECCLES and FATT 1955 a, ECCLES 1957). It can be seen that this positive overshoot was smaller in B than after the spontaneously occurring spike in H, and, when the primary afferent group I volley was increased in C—E, it was replaced by a depolarization. The interpretation of this finding is that the excitatory transmitter substance liberated by the presynaptic terminals "survived" the spike and was able to continue its depolarizing action thereafter. The dorsal root record showed that the group I volley was maximal in E. Further increase of stimulus strength in order to activate group II fibres increased the EPSP in the wake of the spike, as shown by the distinctive hump beginning at the arrow in F, and the second spike arising therefrom in G.

2. The spike potential in deteriorating cells.

The spike potentials described in the last section had the usual characteristic of being and all-or-nothing response. Some cells which immediately after penetration gave this all-or-nothing spike were found to give a graded response when later they had deteriorated, as evidenced from the decreased membrane potential. Records from such a cell are shown in Fig. 3. The potentials in B and partly also in C may be EPSP's but with increase of stimulus strengths in D—H the superimposed graded response has the time course of a spike potential. A possible explanation is that in the initial segment and the cell body the spike generating mechanism is disordered, but impulses still can be generated in the dendrites directly by the EPSP and electrotonically spread to the cell body from which it is assumed that the records were being taken.

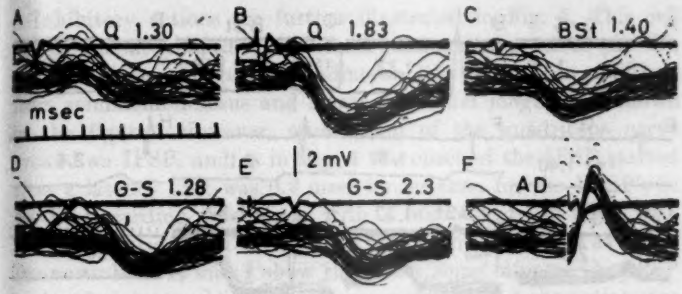

Fig. 4. Simultaneous recording intracellularly from a Clarke's column cell and from the sixth lumbar dorsal root entry zone. In the triphasic records negativity is signalled by a downwards deflection. Stimulation of the nerve to quadriceps (Q) in A, B, of the nerve to posterior biceps-semitendinosus (BST) in C and of the nerve to gastrocnemius-soleus (GS) in D, E. Stimulus strengths relative to threshold strength are indicated in the records. F shows the partial antidromic invasion resulting in stimulation of the dissected Flechsig's fasciculus. About 40 traces are superimposed in each record.

It should be recalled that in Clarke's column the monosynaptic connections from muscle afferents are exerted through giant synapses with long parallel contacts with the dendrites (SZENTÁGOTHAI and ALBERT 1955). This graded spike response has been found only in deteriorating cells, which thus exhibit a behaviour not unlike motoneurones undergoing the chromatolytic reaction (ECCLES, LIBET and YOUNG 1958).

3. Inhibitory actions.

In motoneurones, inhibition is known to be caused by a brief hyperpolarization, the inhibitory post-synaptic potential (IPSP) (BROCK *et al.* 1952). With the inhibitory actions exerted both by Ia and Ib afferents the IPSP begins after a minimal latency that is 0.8 msec longer than that found for the monosynaptic EPSP evoked by impulses in Ia afferents (ECCLES *et al.* 1956, 1957). There is strong evidence that this latency difference is due to the existence of an interneurone in the inhibitory pathway (ECCLES, FATT and LANDGREN 1956, R. M. ECCLES and LUNDBERG 1958). The neurones of the dorsal spino-cerebellar tract also receive inhibitory action by group I afferents, as evidenced by inhibitory effects exerted by conditioning volleys on an excitatory test volley or by the effects of adequate activation of the appropriate receptor organs on a background spontaneous discharge (LAPORTE *et al.* 1956 b, c, LUNDBERG *et al.* 1956). In dorsal spino-cerebellar tract neurones this inhibition is exerted mainly from the muscle

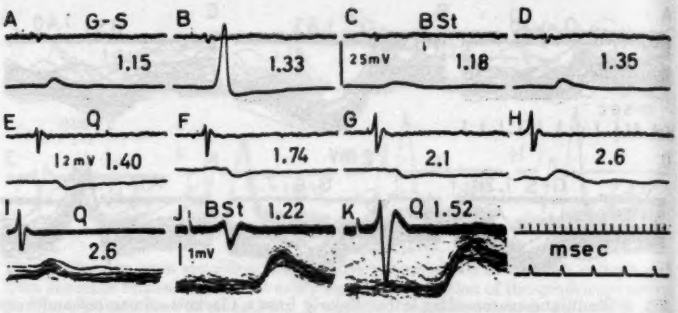

Fig. 5. The lower traces show intracellular records from a cell in Clarke's column and the upper traces the incoming volley recorded at the L VI dorsal root entry zone. In the triphasic records negativity is signalled by a downwards deflection. Stimulation of the nerve to gastrocnemius-soleus (GS) in A—B, and of the nerve to posterior biceps-semitendinosus in C, D, J, and of the nerve to quadriceps in E—H, I, K. Stimulus strengths relative to threshold strength are indicated in the records. Calibration for A—D to the left of record C, for E—I in record E, and J—K in record J. Superimposed traces in record I—K. The upper time signal belonged to all records except J and K.

antagonistic to the one providing maximal excitation, but also from other muscles.

It was of great interest to investigate the mechanism of inhibition, as well as its synaptic linkage. Fig. 4 shows intracelilular records from a cell in Clarke's column. None of the nerves mounted for stimulation had any strong excitatory action, but a small monosynaptic EPSP was evoked by the volley from quadriceps nerve in A and B. The major action by the volley from quadriceps was, however, later a hyperpolarization. A similar IPSP was evoked also by group I volleys from the posterior biceps-semitendinosus (C) and from the gastrocnemius-soleus nerves (D—E). The latency for the onset of the IPSP in B—E relative to the dorsal root volley was 2.3 msec, whereas the corresponding value for the monosynaptic EPSP in A and B was 1.5 msec. This difference of 0.8 msec is exactly that found between the onsets of the EPSP and IPSP in motoneurones (Brock *et al.* 1952, Eccles *et al.* 1956, Eccles *et al.* 1957). On the basis of the assumptions that the conduction times and synaptic delays of excitatory and inhibitory pathways are comparable with those observed for motoneurones, it may be concluded that an interneurone is interpolated also in the inhibitory pathway to the cells of Clarke's column.

Inhibitory actions are further illustrated in Fig. 5. This cell received monosynaptic EPSP's from gastrocnemius-soleus (A—B), from posterior biceps-semitendinosus (C—D) and in addition from semimembranosus and flexor digitorum longus (not shown in the figure). However, stimulation of the quadriceps nerve evoked an IPSP, and as in Fig. 4 the onset of the IPSP started after a latency that was 0.8 msec longer than for the EPSP's in A—D. Recording was made with a micro-electrode filled with 3 M KCl solution, and this cell did not deteriorate as fast as usual. The records in H and I show that after some minutes the IPSP evoked by the quadriceps volley reversed, so that the action was now a depolarization. The superimposed records, J and K, were taken at higher amplification and faster sweep speed in order to reveal the latency difference of 0.8 msec from the nerve to posterior biceps-semitendinosus (J) and reversed IPSP from quadriceps (K). On several occasions it was found that in Clarke's cells the reversed IPSP's could evoke impulses.

Discussion.

Despite the precautions to reduce movement in the spinal cord it was found much more difficult to record intracellularly from Clarke's column cells than from motoneurones. Presumably this is due to some structural difference, since Clarke's cells are only slightly smaller than motoneurones and electrodes of similar resistance have successfully impaled motoneurones for long periods. Despite these difficulties this investigation has given some new results of interest, both with regard to the mechanism of the synaptic action, and on aspects of functional organization.

As in motoneurones, excitatory synaptic action produces a brief depolarization, the EPSP. In some Clarke cells this EPSP could clearly be seen to continue beyond the spike potential, so giving evidence of prolonged transmitter action, which was much less evident with monosynaptic excitation of motoneurones (Curtis and Eccles 1958). This finding provides an explanation of the double spike discharge found with intra-axonal recording from the dorsal spino-cerebellar tract on submaximal stimulation of group I afferents (Laporte *et al.* 1956).

The composite nature of the spike recorded from the cells in Clarke's column parallels the situation in the motoneurone. A

two-stage invasion was originally found with antidromic stimulation (BROCK *et al.* 1952) and was later recognized also during orthodromic activation (ARAKI *et al.* 1955, FUORTES *et al.* 1957 a, b, COOMBS *et al.* 1957 a, b). From the analysis made on motoneurones there is strong evidence that this two-stage invasion signifies a higher threshold for excitation in the soma than in the initial segment and that with orthodromic activation the impulse is generated in the initial segment (ARAKI *et al.* 1955, FUORTES *et al.* 1957, COOMBS *et al.* 1957 a, b).

Another similarity with motoneurones (cf. BROCK *et al.* 1952) is provided by the fact that also in Clarke's cells inhibitory action is exerted by a brief hyperpolarization, the IPSP. Furthermore since this IPSP, as in motoneurones (COOMBS *et al.* 1955), reverses after some time when recording is made with electrodes filled with KCl solution, it is likely that the same ionic mechanism is utilized to evoke this hyperpolarization. Of particular interest is the finding that also in Clarke's cells the onset of the IPSP occurs after a latency 0.8 msec longer than that found for the monosynaptic EPSP. Since this longer latency in the motoneurone is due to the existence of an interneurone in the inhibitory pathway (ECCLES *et al.* 1956, R. M. ECCLES *et al.* 1958), we may infer that this is also so in the present case, thus providing support for the generalizing suggestion of ECCLES *et al.* (1956), that an interneurone exists in all inhibitory pathways. It is of interest that the inhibition from other sources than the antagonist also is evoked through a disynaptically relayed IPSP and is produced by group I impulses. An alternative mechanism for producing inhibition of the background discharge could have been a cessation of the excitatory bombardment maintaining the resting discharge in these cells (cf. HOLMQVIST *et al.* 1956).

Summary.

1. Intracellular recording from the neurones of Clarke's column shows that the cells suffer much more from the impalement of the micro-electrode, as evidenced by a rapid decline of the membrane potential. It has been possible to show that in general these neurones resemble motoneurones in their electrical responses.

2. With antidromic as well as with orthodromic activation the spike potential has two components. On analogy with the motoneu-

rone it is inferred that the soma has a higher threshold than the initial segment and that on orthodromic activation the impulse is initiated in the initial segment. Excitatory and inhibitory synaptic actions evoke depolarizing and hyperpolarizing post-synaptic potentials respectively.

3. Reversal of the inhibitory post-synaptic potential was found when micro-electrodes filled with a KCl solution were used for recording, suggesting that the ionic mechanism of its production is the same as in motoneurones. The onset of the inhibitory post-synaptic potential had a latency that was 0.8 msec longer than that of the monosynaptic excitatory post-synaptic potential. It is concluded that an interneurone is interpolated in the inhibitory pathway to the cells in Clarke's column, just as occurs with motoneurones.

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Membrane and Action Potentials of Spinal Interneurons in the Cat.

By

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Introduction.

The intracellular microelectrode technique has in the last years been applied in various investigations of spinal interneuron functions. The criteria for the identification of interneurons have been outlined and the potential characteristics of these neurons analysed by WOODBURY and PATTON (1952) and by FRANK and FUORTES (1955). The latter authors presented a rather extensive discussion of the intracellular recording technique as applied to the spinal cord and dealt especially with the problem of the origin of the action potentials recorded. On the other hand, the rhythmical activity and the functional organization of different types of interneurons have been the main subjects of other investigations (KOLMODIN and SKOGLUND 1954, ECCLES, FATT and KOKETSU 1954, ECCLES, FATT and LANDGREN 1954, 1956, FRANK and FUORTES 1956, KOLMODIN 1956, SKOGLUND and KOLMODIN 1956, KOLMODIN 1957; for a more complete review of these and other papers dealing with spinal interneuron functions see the latter work).

The present paper gives an account of the experimental technique used in the series of experiments we have carried out during the last years. In the first section results from continuous recordings of the membrane potential level in interneurons are presented and

the absolute value and the stability of this level are compared with corresponding data obtained from afferent fibres and motoneurons. The second section gives a description in more detail than in our previous reports of the characteristic responses of interneurons to synchronous afferent stimulation; typical records from spontaneous and naturally induced activity are also presented. Special attention is given to the prepotential and firing level variations and their relation to the membrane potential level.

Methods.

Preparation. Decapitate cats kept on artificial respiration were used. The animals were curarized but control experiments without curarization were also made. In most experiments the ipsilateral ventral roots L_4—S_1 were cut and, one by one, placed on electrodes for antidromic stimulation. The dorsal roots were usually left intact except in a few experiments in which electrical stimulation was applied to them. For artificial nerve stimulation skin or muscle nerves were dissected free and kept under paraffin. When natural stimulation was used, the hindlimbs were usually kept intact, or only the tendons of the muscles which were to be subjected to stretch were dissected free, and necessary operations for fixation made.

The animal was mounted in a holder allowing of rigid fixation particularly in the region of the exposed lumbar cord; on the same stand a Zeiss micromanipulator was mounted, by means of which the microelectrode could be slowly pushed into the cord through a hole dissected in the pia arachnoidea. The various depths from which the recording was made could be read off on a micrometer gauge attached to the micromanipulator. As a rule only one electrode was inserted into each segment of the cord in order to avoid too extensive injury. When a recording was finished the electrode used was left in its position in the cord. At the end of the experiment the cord was fixed *in situ* by intravenous injection of a 10 per cent formalin solution; it was also covered with the same solution. The preparation was then left untouched in the holder until, after 12—24 hours, the cord segments were carefully dissected free. After further fixation *in vitro* serial frozen sections of a thickness of 50 μ were made, in which the electrode track could easily be found and the position of the electrode tip at the various

recording points determined by means of the values obtained from the micrometer gauge. Taking into account obvious sources of error, *e. g.* shrinkage due to fixation, the accuracy of this determination is not claimed to be more than $\pm$ 100 μ.

Microelectrodes. The capillary electrodes were produced by means of an automatically operating pulling device similar to that described by ALEXANDER and NASTUK (1953). The shape and the tip diameter of the capillary obtained by this method obviously depend on a number of factors, such as the properties and dimensions of the glass tubes employed, the length, diameter and number of turns of the heating coil and its working temperature, the stability of the heating current and further the initial and final pulling forces. No doubt many different combinations of values of these factors may give satisfactory results, but our own data may be worth mentioning. Pyrex glass tubes of 1.8—1.9 mm outer and 1.25—1.35 mm inner diameter were used. The heater coil which consisted of 7 turns of a platinum wire, 0.8 mm in diameter, had an inner diameter of 3 mm and a length of 7 mm. The initial pulling force was 100 g and the final pulling force approximately 1,500 g. A lower value of the initial pulling force was observed to tend to make the adjustment of the heater coil temperature critical. According to our experience the taping of the electrode tip is of great significance for the recording of stable membrane potentials, electrodes with too steep taping thus being less suitable.

Several ways of filling the micropipettes were tried before the method of boiling in alcohol under reduced pressure was finally adopted (TASAKI, POLLEY and ORREGO 1954). The electrodes were kept in 2.7 M KCl solution in order to minimize the formation of KCl crystals at the tip — a drawback often encountered when using 3 M solution. The microelectrodes were seldom stored more than 5 days in the KCl solution.

The electrical resistance of the microelectrodes was roughly checked by a vacuum tube testmeter provided with a 0—1,000 megohm range, the tip of the electrode being immersed in Ringer's solution. Sometimes, however, widely varying values were obtained on repeated measurements. One possible explanation might be a drifting of particles in and out of the tip, a phenomenon actually observed. This movement might be caused by the current flowing through the electrode, the value of the current being approximately 10^{-7} amperes when measuring a resistance of 10

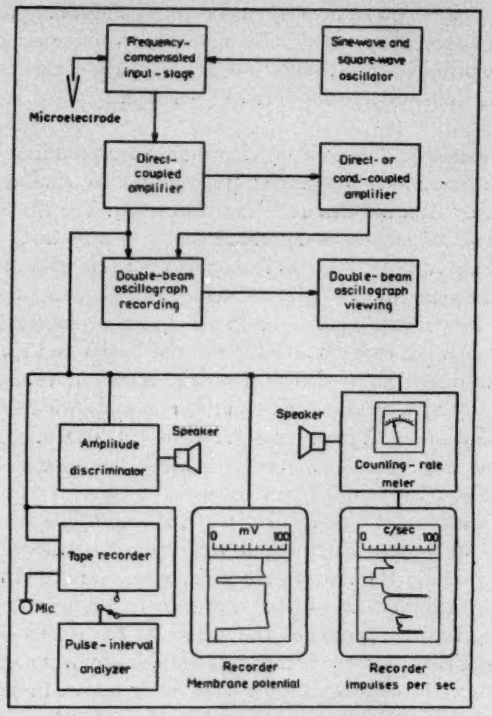

Fig. 1. Block diagram of recording equipment.

megohms. A more accurate method for determination of the resistance was therefore developed (see below).

Recording devices. The arrangement of the recording equipment used in these investigations is shown in the block diagram Fig. 1.

The capillary microelectrode is connected to an input-unit of cathode follower type, in which positive feedback is employed to counteract the influence of various inevitable stray capacitances and thus to bring about an improvement in high-frequency response. The input-unit has been described in full detail elsewhere (HAAPANEN and OTTOSON 1954).

Immediately before insertion of the microelectrode into the preparation the resistance of the electrode was measured by means

of alternating current, supplied by a variable frequency oscillator of constant amplitude and applied between the electrode tip and ground. Only the utmost tip of the electrode was then in contact with the thin Ringer layer covering the spinal cord of the cat grounded only through the variable frequency oscillator. A voltage division takes place over the series combination of electrode resistance and the amplifier input capacitance, the positive feedback control for frequency compensation being turned off. The frequency of the test oscillator is then increased until the amplitude of the recorded signal drops to 70 per cent (−3 dB) of its value at 15 c/s, the lowest frequency supplied by the oscillator. At the higher frequency the value of the resistance equals that of the capacitive reactance, which is known, and a table can be prepared from which the resistance is read off. In those cases when the microelectrode was withdrawn from the preparation a new check on the electrode resistance was usually made. The resistances thus measured of the electrodes used in the present investigations have varied between 15 and 50 megohms.

Next, adjustment of the feedback control of the input stage was made for best "square-wave response" and finally a check on the frequency response to a sine-wave made by means of the signal generator. The frequency at which this response decreased to 70 per cent (−3 dB) of the low frequency value was usually in excess of 5,000 cycles, implying a recording time constant of about 30 μsec or less.

When the electrode had been brought into recording position at the surface of the spinal cord, the amount of feedback was again checked by the square-wave response and correction made for the shunting effect of the capacitance across the shaft of the electrode. Upon advancement of the electrode the square-wave test was repeated at approximately every 0.5 mm.

It was observed that when the tip of the electrode was located 4—5 mm within Ringer's solution or the spinal cord and the feedback correctly set, a continued advancement of the electrode required no further adjustment of the feedback control. The frequency of the recording system was thus kept under continuous control and overcompensation was not allowed to develop. If overcompensated, our input amplifier may record a spike potential at about 20 per cent too high an amplitude.

The signal from the input-unit is then amplified in a direct coupled amplifier (HAAPANEN 1953), the output of which deflects

one of the cathode rays of a double-beam oscillograph and also operates several recording instruments, *i. e.* a pen recorder (frequency response 1/sec) for the resting potential, a counting rate meter and pen recorder (frequency response 1/sec) indicating the frequency of the impulses, and a tape recorder. The pulse-interval analyser described elsewhere (HAAPANEN and SKOGLUND 1955) can be connected to the output of either the amplifier or the tape recorder.

By further amplification in a condenser-coupled amplifier the action potential is magnified for closer examination and displayed on the second cathode ray.

Although incidentally not used in the present investigation, the rate meter belonging to the recording apparatus is included in the block diagram Fig. 1. It is of the type used in radiation measurements (cf. ELMORE and SANDS 1949), amended so as to suit our purposes. Thus, by adding an amplitude discriminator the responses can be limited only to impulses exceeding a preset amplitude. The instrument is further designed so as to be paralyzed for 0.9 msec after an impulse has arrived. The lowest range chosen is 0—50 and the highest 0—1,000 pulses per sec, which has proved to be satisfactory. The time constant of the integrating circuit in the rate meter was generally kept low enough to make the response time of the pen recorder (1/sec) the limiting factor.

In order to minimize current spread in the preparation and reduce pick-up of artefacts the stimulators are provided with radio frequency coupled isolation units which are especially valuable when stimulating root filaments; for the recording from roots, radio frequency coupled input stages are also employed (cf. HAAPANEN 1952, HAAPANEN, HYDE and SKOGLUND 1953).

Results.

I. Membrane potential levels of interneurons as compared with such of afferent fibres and motoneurons.

In the standard procedure used in our experiments, a zero potential line has first been established by lowering the electrode into the layer of Ringer's solution covering the cord surface; the electrode is then slowly pushed further downward by minute movements of the micrometer screw, alternating with light tapping on the electrode holder until potential changes appear.

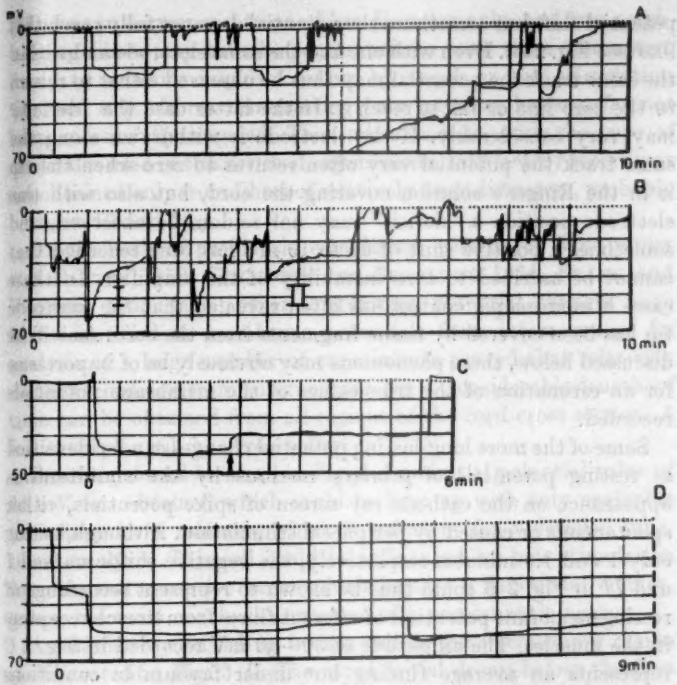

Fig. 2. *A* and *B*, continuous recordings of membrane potential shifts occurring along electrode tracks in the dorsal column; *C*, continuous recording of membrane potential from afferent fibre in the dorsal root. At *arrow* slight tapping on the micromanipulator. *D*, similar recording from afferent to the gastrocnemius muscle. See text. *Time between vertical lines* 30 sec in this and the two following figures. Time in minutes represents total period of continuous recording.

When the electrode comes into contact with the cord surface small positive deviations of maximum 5—10 mV are sometimes observed. As the electrode is pushed on downward sudden negative shifts are recorded which vary in amplitude from a few mV up to 70 mV, as may be seen in Fig. 2 *A* which represents a continuous record of the potential shifts which occurred within the first mm of an electrode track in the dorsal column. Some of these potentials are transient, lasting only a few seconds; others maintain their initial level for a longer period followed by a rapid return to the baseline, while still others display a slower, successive decline from the initial value. Usually there is a return close to the zero line between each

potential shift but sometimes the potential does not fully reach this line (see Fig. 2 *B*). Even with one and the same electrode and within the same track the potential may thus be observed either to return to the zero line or fail to reach it. In the latter case the "deficit" may vary considerably. If the electrode is withdrawn along the same track the potential very often returns to zero when the tip is in the Ringer's solution covering the cord, but also with this electrode position a "deficit" may not seldom be observed, and sometimes a positive shift of up to 10 mV has been recorded that cannot be ascribed to zero instability of the amplifier. In these cases a microscopic control has often revealed that the electrode tip has been covered by tissue fragments from the cord. As will be discussed below, these phenomena may obviously be of importance for an estimation of the true values of the membrane potentials recorded.

Some of the more long-lasting potential changes can be identified as resting potentials of primary neurons by the simultaneous appearance on the cathode ray screen of spike potentials, either spontaneous or caused by peripheral stimulation. Although lasting only 1 and 1.5 minutes respectively, the negative shifts marked *I* and *II* in Fig. 2 *B* could thus be shown to represent recordings of resting membrane potentials of afferent fibres from stretch receptors in the muscles. The amplitude of 30—40 mV recorded in Fig. 2 *C* represents an average finding but under favourable conditions higher values may be obtained and also maintained for a considerable time. An example is given in Fig. 2 *D*; the resting potential of this fibre increased stepwise and somewhat sluggishly — as sometimes happens — to a plateau between 50 and 60 mV. The slow decline during the first five minutes could be counteracted and the resting potential restored by a slight tapping on the electrode holder, but after five minutes more the lower level of 50 mV was reached again. This phenomenon — which has also been observed when recording from other spinal elements — may in part be explained by the electrode being pushed slightly further on into the axon during the tapping, thus better sealing the hole in the membrane caused by it, but there may also be other explanations (cf. Discussion).

Most of the fibres identified proved to be muscle afferents; in one and the same run recordings are often made from afferents originating in different muscles. The movement necessary to change the electrode position from one fibre into another is often so small

(15 μ) as to correspond to one fibre diameter only, and two neighbouring fibres may thus be penetrated. In some cases fibres were found to transmit impulses set up by touch or hair stimulation whereas, *e. g.*, fibres transmitting pain impulses have so far not been successfully impaled. This indicates that, with the type of electrodes used in this series of experiments, satisfactory recordings are obtained only from fibres of relatively large diameters, probably between 10 and 20 μ.

On the basis of previously established criteria (cf. WOODBURY and PATTON 1952, KOLMODIN and SKOGLUND 1954, FRANK and FUORTES 1955, 1956) other long-lasting potential changes have been identified as membrane potentials from interneurons. An analysis of a large number of experiments proved that relatively stable recordings from interneurons for a considerable length of time can be obtained from all regions of the cord cross section. A few examples will be given below.

From the *dorsal horn* cell in Fig. 3 *A* an initial potential value of 74 mV was obtained which could be kept up with only negligible variations for 6 minutes, which is sufficient for a detailed functional analysis. This cell could be influenced by hair stimulation from several receptive areas which implies a functional convergence, the most conclusive criterion of a postsynaptic neuron.

A satisfactory recording from a cell in the *intermediary* region is illustrated in Fig. 3 *B*. The rapid initial decay before the first steady level of 50 mV is reached is a common finding. The final plateau of 40 mV was very stable; the recording was discontinued on purpose after 14 minutes. This lowering of the recorded membrane potential value was not accompanied by any signs of functional deterioration of the cell; the initial discharge frequency as well as the pattern of activation remained unchanged.

In the next example, Fig. 3 *C* — a cell from the *commissural* region — there was a more pronounced decrease of the membrane potential, amounting to about 50 per cent in 15 minutes, but in spite of this the discharge pattern was not affected. Other examples of recordings from commissure cells are given in Fig. 3 *D*. These two cells were situated 200 μ apart, and in this case the zero level was not attained between the two impalements.

As a final example a record from a *ventral horn* interneuron is presented in Fig. 3 *E*. The maintenance of a level of 40 mV after 70 minutes of recording is an exceptionally good result for interneurons.

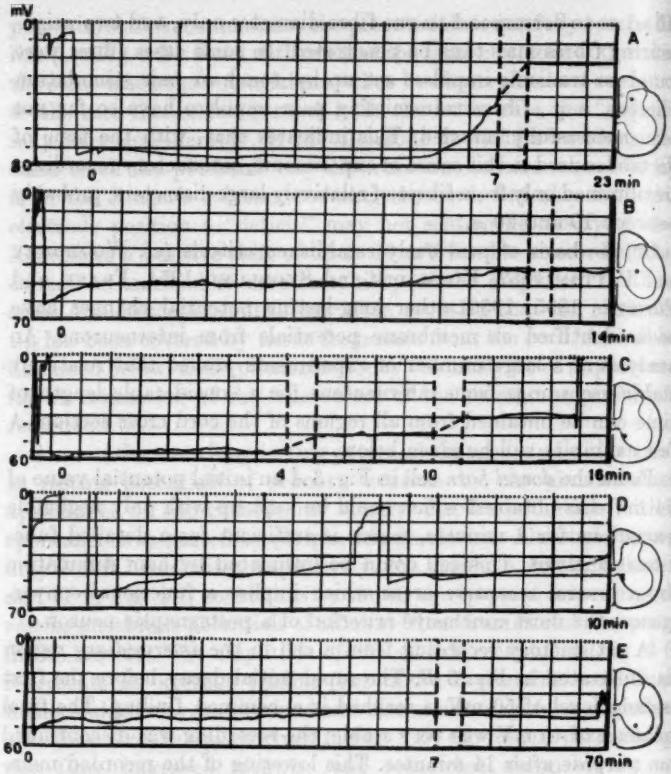

Fig. 3. Continuous membrane potential recordings from interneurons in various parts of the cord cross section as indicated by diagrams to the right. Parts of recordings cut out (between *dotted vertical lines*). See text.

The highest membrane potential value recorded from interneurons has been 80 mV, the average being 40 mV. This value is based on the relatively stable level of the membrane potential. The maximal duration of a recording has been 70 minutes, and the average recording time for the experiments above was about 10 minutes.

In many interneurons there are spontaneous, more or less regular membrane potential fluctuations of up to 10 mV amplitude. These fluctuations are more common and their amplitude is larger in neurons that are on the verge of activity or have a low threshold

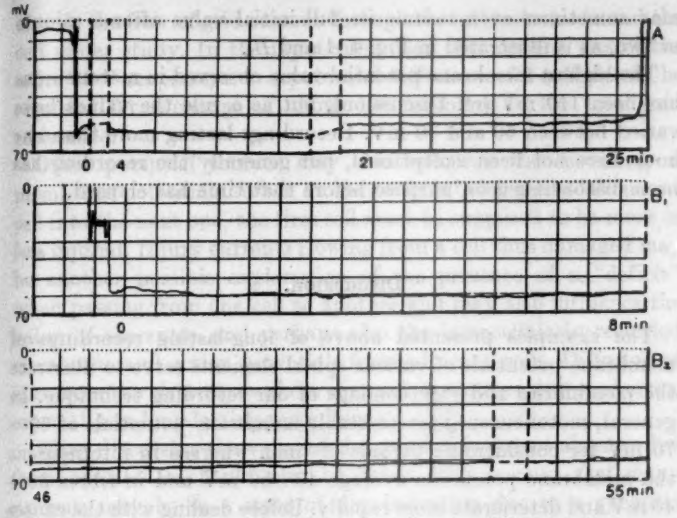

Fig. 4. Continuous membrane potential recordings from two different motoneurons, *A* and B_1—B_2. Parts of recordings cut out (between *dotted vertical lines*).

for sensory stimulation. In some neurons sensory stimulation may cause an evident, more or less long-lasting depolarization or an increase of the amplitude of the membrane potential fluctuations with or without a discharge of spikes.

In *motoneurons*, on the other hand, long-lasting recordings of stable high-amplitude membrane potentials can readily be obtained, as is well known from the work of ECCLES and co-workers. As a comparison with the pictures from interneurons, a characteristic example of motoneuron membrane potentials is given in Fig. 4 *A*. The next records, B_1—B_2, give a further illustration of the extreme stability of these neurons which is so striking that even before the final criterion has been established — *viz.* the typical antidromic response to ventral root stimulation — there is often no doubt as to which type of unit has been impaled.

As shown in Fig. 3 the initial membrane potential recorded from interneurons was usually found to decline 5—10 mV during the first 15—30 sec before reaching the comparatively steady level. The decline of the initially recorded membrane potentials is much less pronounced in motoneurons whose membrane potential

may sometimes even resume its full initial value after a minute or two, as is illustrated in Fig. 4 *A* and B_1.

The highest membrane potential value observed in motoneurons has been 110 mV (cf. Discussion), but as a rule the values have varied between 60 and 70 mV. Recordings lasting more than one hour have not been exceptional, but generally the recording has been discontinued on purpose before that time has elapsed.

Discussion.

The examples presented above of long-lasting recordings of membrane potentials of various spinal elements serve to illustrate the possibilities and shortcomings of our recording technique. In general, motoneurons present stable membrane potentials of 60—70 mV for considerable periods of time, whereas in interneurons the membrane potential is average 40—50 mV and in fibres 30—40 mV and deteriorate more rapidly. Before dealing with the causes of these differences it is necessary to discuss the various factors influencing the values of the recorded membrane potentials, since observations made in the present study suggest that other potential sources than the membrane of the particular cell under study may have been involved in the production of the recorded potential.

One possible potential source of this kind is the appearance or disappearance of a contact potential between the electrolyte of the electrode and the nervous matter. The significance of contact potentials in microelectrode recordings has so far been discussed chiefly in connection with studies on muscle and nerve fibres (cf., *e. g.*, CURTIS and COLE 1942, NASTUK and HODGKIN 1950, CASTILLO and KATZ 1955, ADRIAN 1956). When recordings have been made from the central nervous system the problem has met with far less interest. In the present investigation it has not been possible to analyse contact potentials of this kind more closely but it seems reasonable to assume that phenomena such as "deficit" (*viz.* that the zero line is not attained when the electrode passes from one cell into another), instability of the zero line and probably also the stepwise changes of the membrane potential during impalement of some neurons should, at least in part, be explained as being caused by contact potentials between the electrolyte within the electrode and the external medium, *e. g.* Ringer's solution, cytoplasm or injured fractions of nervous tissue. Another obvious source of

error is the flow of grid current through the microelectrode and the cell under study. In the input tube used this current was of the order 10^{-11} amperes, *i. e.* a potential difference of 1 mV should be recorded for a change of about 100 megohms in the total grid resistance.

Other potential sources may also influence the recording of membrane potentials. When an electrode is being pushed from one cell into the next one, the first cell must be supposed to be more or less injured. Injury currents flowing from a cell thus damaged may be another possible explanation of the presence of a "deficit" when passing from one cell to another and may also influence the value of the extra- and perhaps also the intracellularly recorded membrane and action potentials from adjacent cells. Diffusion of ions from the electrolyte of the electrode is another source of error, especially in long-lasting recordings.

When discussing the value of the membrane potentials recorded from somata it should also be taken into account that many neurons may be in a state of depolarization due to a synaptic bombardment from interneurons or afferent fibres. A physiological reduction of this kind in the value of the membrane potential may amount to about 15 mV and is more common in non-anesthetized than in anesthetized preparations (cf. also KOLMODIN and SKOGLUND 1958).

It is generally accepted that mechanical injury of the cell membrane may result in low voltage or deteriorating membrane potentials. A common phenomenon in the experiments described above has been the presence of an initial higher membrane potential immediately after the impalement which declines and is later stabilized at a lower level (see Fig. 3). Such a deterioration has been more common and its value has been higher the smaller the penetrated elements were supposed to be; in motoneurons the initial value of the membrane potential was sometimes regained (Fig. 4). A certain relationship thus seems to exist between the values of the recorded membrane potentials and the degree of injury caused to the cells (cf. also WOODBURY, HECHT and CHRISTOPHERSON 1951).

In the following an attempt has been made to find a relation between the degree of injury, cell size and recorded membrane potential, starting from certain assumptions. It is, *e. g.*, very likely that the injury caused by the microelectrode — minute as it may be — manifests itself as an abolished or decreased membrane

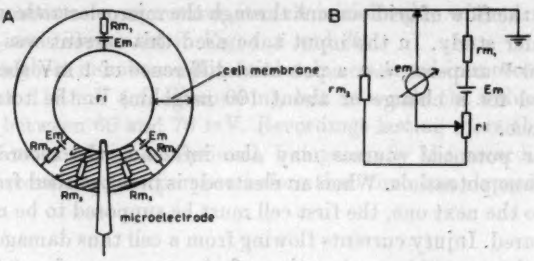

Fig. 5. Electrical circuits equivalent to situation during intracellular recording. Full description in text.

resting potential across the damaged area around the electrode. As soon as the membrane potential in the damaged part of the membrane decreases the undamaged membrane area starts supplying current through the damaged area. We have thus a closed loop involving a number of resistances; the "unloaded" membrane potential is divided on these resistances according to their real values. Although the degree of injury around the electrode is likely to have a gradient, the membrane can be thought of as consisting of one fully undamaged part and one part in which the breakdown is complete. This situation is shown in the diagram in Fig. 5 *A*.

In the figure, Rm_1 and Rm_2 represent the membrane resistance per unit area in the undamaged and the damaged parts of the membrane respectively, the shaded area representing the injured part lacking membrane potential *Em; ri* is the resistance of the cytoplasm. The membrane capacitance is neglected because only the steady state of the cell is considered. When all resistance elements Rm_1 and Rm_2 in Fig. 5 *A* are represented by the total resistance rm_1 and rm_2, the electrical diagram shown in Fig. 5 *B* results. The recorded potential *em* is seen to be only a part of the full potential *Em*, the value being dependent on the voltage division between rm_1 and rm_2. Most authors agree that the resistance of the cytoplasm (*ri*) is very low (*e. g.* ARAKI and OTANI 1955, ECCLES 1957); *ri* may therefore be neglected. However, this argumentation cannot apply to structures of a considerable length, like axons, where the inner resistance cannot be neglected in the same manner (cf. ECCLES 1957).

There is reason to assume that the injury in the membrane is accompanied not only by an abolished or decreased membrane

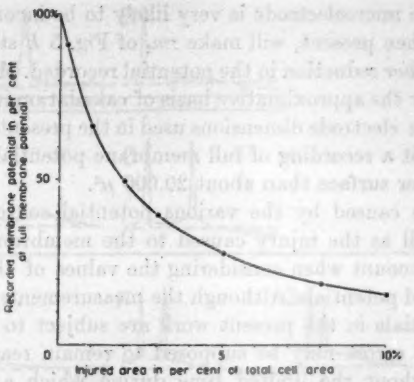

Fig. 6. Relation between calculated recorded membrane potential and degree of injury. See text.

potential but also by a decreased membrane resistance. No values of this drop in resistance are available but it may be assumed that such a decrement would be of the same order as the change in resistance during the passage of an impulse in nerve fibres (COLE and CURTIS 1939, TASAKI 1955).

The curve in Fig. 6 is based on the assumption that the membrane potential in the injured area is abolished and that the resistance of this area has dropped to 2 per cent of the original value. The injured area is given as a percentage of the total membrane area and the recorded membrane potential in per cent of full membrane potential. For example, an electrode inserted 3 μ into a motoneuron with a diameter of 70 μ and a calculated surface area of 50.000 μ^2 (ECCLES 1957) will make a hole in the membrane of a diameter of 1.5—2.5 μ, and if the damaged area around the electrode is estimated to be only 2 μ wide the total damaged area will amount to about 30 μ^2 which is 0.06 per cent of the total surface area. As appears from the curve in Fig. 6 the reduction of the membrane potential in that case is negligible. If, on the other hand, an interneuron with a diameter of 30 μ and a calculated surface of approximately 10.000 μ^2 is penetrated by the same electrode, the damaged area of 30 μ^2 will constitute 0.3 per cent of the calculated surface area. Such a damage will, according to the curve, reduce the membrane potential by about 15 per cent.

In this calculation we have not taken into account that the

sealing of the microelectrode is very likely to be incomplete. Such a leakage, when present, will make rm_2 of Fig. 5 *B* still lower and lead to a further reduction in the potential recorded. Making proper allowance for the approximative basis of calculation it seems probable that the electrode dimensions used in the present experiments do not permit a recording of full membrane potentials from units with a smaller surface than about 20.000 μ^2.

The errors caused by the various potential sources discussed above as well as the injury caused to the membrane should be taken into account when considering the values of the intracellularly recorded potentials. Although the measurements of the membrane potentials in the present work are subject to quantitative errors, these errors may be supposed to remain reasonably constant throughout the limited time during which a stable d. c. potential is recorded from one and the same cell with the electrode in a constant position. During such periods it is possible to study physiological variations of the membrane potential level not only in motoneurons but also in interneurons, as will be exemplified in the next section and also in a more detailed analysis in a following paper (KOLMODIN and SKOGLUND 1958).

II. Action potentials from interneurons.

Responses to synchronous afferent volleys. As has been described previously (KOLMODIN and SKOGLUND 1954), the interneuron response to single shock stimulation of a peripheral nerve or a dorsal root may be a single spike or — more often — a burst of impulses. Similar observations were made by FRANK and FUORTES (1956) who also found that certain interneurons responded by a discharge consisting of a train of impulses composed of more than 100 impulses and lasting more than 1 sec. This has been confirmed in our experiments in which also discharges consisting of a burst of impulses followed by single, irregularly spaced impulses during several seconds have been observed.

In order to illustrate one common type of interneuron response to electrical stimulation we have selected the series of records in Fig. 7, obtained from an interneuron in the ventral part of the cord activated by dorsal root stimulation. Its resting potential kept constant at 70 mV for more than half an hour.

In Fig. 7 *A*, taken with faster sweep speed than the following recordings, threshold stimulus strength for eliciting a response was used. After a latency of 3.5 msec a slow rise of the baseline is seen

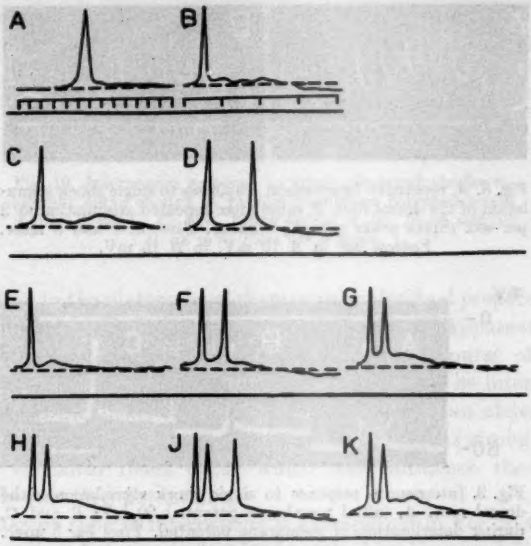

Fig. 7. Interneuron responses to electrical dorsal root stimulation of increasing strength. Membrane potential level 70 mV marked by *dotted line*. Maximum spike potential 50 mV. *A*, time in msec; *B*, time in 5 msec; *C*—*K* same sweep speed as in *B*. Full description in text.

and on this "prepotential" the spike takes off after a further delay of about 1 msec. The spike potential rises to its peak in 0.5 msec and its duration is 1.6 msec. The spike is followed by a slower potential change so that in this record the original resting potential level is not attained until after about 5 msec. As appears from record *B*, taken with the same stimulus strength on a more compressed time scale, a sequence of irregular potential changes may follow. When the stimulus strength is slightly increased, these slow potential changes become more pronounced (*C*), and their "prepotential" nature is obvious when a second spike is also fired on the crest of such a potential (*D*). At this stimulus strength the interval between the first and second spikes was long and somewhat varying, but with a further increase in stimulus strength both prepotential and spike move nearer the first spike (*E* and *F*), until a fixed minimum interval is reached (*G*). The prepotentials have now fused into a single plateau of high amplitude and more than 10 msec duration. Keeping the stimulus strength constant (*H*—*K*),

Fig. 8. *A*, repetitive interneuron responses to single shock stimulation of the dorsal root; *B*, same after repeated stimulation at 2 per sec. (Spike peaks cut off.) See text. *Time* in 1 and 5 msec. *Vertical bar* in *A* 10 mV, in *B* 15 mV.

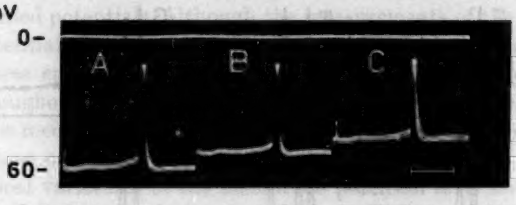

Fig. 9. Interneuron response to single shock stimulation of the dorsal root. *A*, initial membrane potential 60 mV; *B* and *C*, during deterioration of membrane potential. *Time bar* 5 msec.

repeated stimulation every two seconds may lead to somewhat varying effects, apparently reflecting fluctuations in excitability. Thus, while in *H* only two spikes are elicited, a third spike appears in *J* (at about the same latency as the second spike in *D*). At the next stimulus, *K*, it has dropped out again but is now replaced by a small potential hump. It should be noticed that there is a reduction in size of the second spike in this preparation, beginning at an interval of 2 msec.

Even at threshold stimulation some interneurons cannot be brought to fire with merely one spike. Neurons of this highly repetitive type may respond to suprathreshold afferent single shock stimulation by a very high rate of discharge. An example is given in Fig. 8 *A;* in this case the maximal response consists of 9 spikes during 12 msec, corresponding to a frequency of about 750 per sec. After repeated stimulation the later spikes in the series drop out first, sometimes at such a low frequency as 2 per sec (*B*), but even the most resistant first spike seldom followed higher stimulus frequencies than 30 per sec.

These records have also been chosen as illustrative of the typical prepotential configurations during such repetitive discharges;

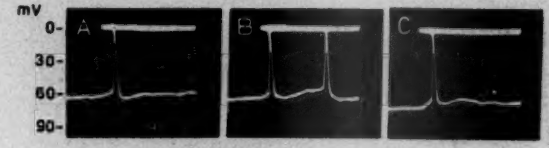

Fig. 10. Interneuron response to single electrical shocks to afferent nerve elicited at various membrane potential levels: *A*, 61; *B*, 62; *C*, 69 mV. Firing levels: *A*, 55; *B*, 56 and 53; *C*, 62 mV. *Time bar* 5 msec.

in addition to the plateau level there occur individual prepotentials, the rate of rise of which varies with the interval. Apparently the firing level also varies considerably during the course of these discharges. However, these potential pictures must be interpreted with a certain caution since they are the result of an abnormally synchronized afferent activity; under such conditions strong extracellular electrical fields occur which may influence the intracellularly recorded potential.

A different type of interneuron is exemplified in Fig. 9 *A*. This neuron responded to single shock orthodromic stimulation by merely one spike and in this type of neuron relatively strong stimuli are, as a rule, necessary to elicit a repetitive discharge. The potential picture has certain similarities to that of a motoneuron activated multisynaptically, with a slowly rising prepotential of low amplitude.

Records *B* and *C* illustrate how in this case the configuration of the whole potential sequence may be maintained in spite of a deteriorating resting potential. In other cases, under similar conditions, a pronounced decrease of the prepotential may be observed, and it is obvious that varying degrees of cell injury may be one factor accounting for the variations in prepotentials occurring from cell to cell (cf. Discussion).

However, even in one and the same neuron, during constant recording conditions, considerable variations may be found in the shape of the prepotentials as well as in the threshold level for the initiation of spikes by afferent single shock stimulation. In Fig. 10 are shown some examples of action potentials of an interneuron where the *firing levels* vary from 53 to 62 mV. As appears from the records, the initial membrane potential level before the onset of synaptic excitation is also varying, from 61 to 69 mV. This led us to examine if there is a relationship between the firing level and the

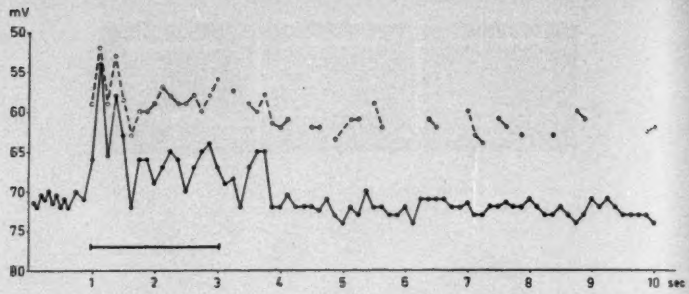

Fig. 11. Graphical representation of variations in membrane potential (*filled circles*) and firing level (*open circles*) as result of application of pad pressure (*horizontal line*). Same interneuron as in Fig. 10. Single shock stimulation of afferent nerve at 8 per sec starts at point marked 1st sec and continues throughout the figure. Filled circle without corresponding open circle indicates absence of response to stimulation.

initial membrane potential level. For this purpose we have made use of the possibility to vary the membrane potential value by means of natural sensory stimulation (cf. KOLMODIN and SKOGLUND 1958) and then, by electrical test shocks to an afferent nerve, elicit action potentials at different membrane potential levels.

An experiment of this type is illustrated in Fig. 11 which is a graphical representation of the values of the initial membrane potential (filled circles) and of the firing level (open circles) obtained from measurements on a series of recordings of 45 consecutive action potentials elicited by electrical stimulation at 8 per sec. In the first part of the curve no afferent activity is induced and the neuron is in non-firing state; the variations of the membrane potentials are then within the range of 2 mV. Pressure is then applied to the pad for about 2 sec (horizontal line) and simultaneously the gastrocnemius nerve is stimulated electrically. It can be seen that the sensory stimulation causes marked fluctuations of the membrane potential in depolarizing direction; the neuron is thus hit by the electrically induced afferent volleys at varying membrane potential levels, and when the firing level is reached spikes are elicited. During and immediately after the sensory stimulation the values of the initial membrane potential as well as of the firing level decrease and there are considerable variations both in the initial membrane potential and in the firing level. After cessation of the sensory stimulation the values of these levels gradually begin to increase again, and after about 3 sec the first case occurs when no spike is elicited by the test stimulation,

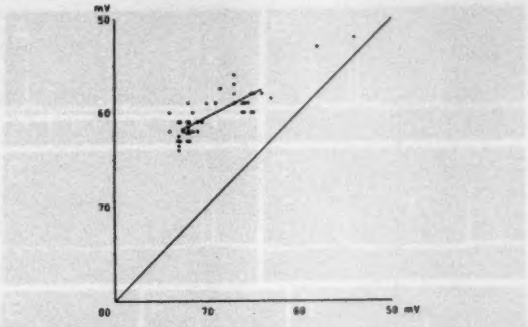

Fig. 12. Y-axis values represent firing levels, x-axis values membrane potentials, both from Fig. 11. Vertical distance from each point to 45° line represents difference between membrane and firing level. Line through scattered points shows the trend and is drawn as per following. The mean x and y values for one-third (15) of the points, those with lowest x values, are calculated. The same is done for the one-third (16) with the highest x values. The grand mean of all points is calculated, and through this value is passed a straight line parallel with the line (not shown) joining the means of the highest and lowest thirds.

as indicated by the absence of open circles. From now on the excitability declines parallel with the increase of the membrane potential until finally, from the 7th to the 9th sec, only 8 out of 16 stimulations result in spikes.

The 45 action potentials represented graphically in Fig. 11 have thus been elicited at random at initial membrane potential levels varying from 74 to 54 mV. In Fig. 12 the corresponding values of the initial membrane potential and the firing level of the spikes have been plotted against each other. It appears from the diagram that the firing level varies within 12 mV, whereas the value of the membrane potential varies within the range of 20 mV. It is also of interest to observe the large variations of the firing level at a given initial membrane potential value. Thus, for instance, 9 spikes have been elicited at an initial membrane potential level of 72 mV and, as shown in the figure, the firing level for these spikes varied between 63 and 59 mV. On the whole there is, however, a marked correlation between membrane potential and firing level, an increased membrane potential corresponding to a higher value of the firing level, and the curves suggest, approximately, a direct proportionality between these two levels.

Using afferent single shock stimulation it is often difficult to analyse the spike after-processes on account of the presence of

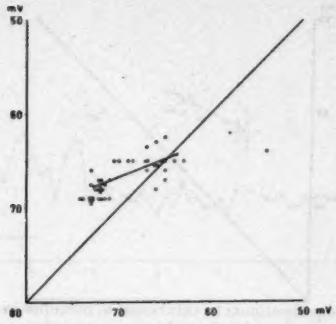

Fig. 13. Y-axis values represent repolarization levels, x-axis values membrane potentials, from the same experiment as Figs. 11 and 12. Trend shown by line derived as in Fig. 12.

prolonged slow potential fluctuations, as exemplified in Fig. 7. In other cases, like that analysed above, the spike *repolarization level* can be more exactly determined. In the experiment described in Figs. 10—11 the values of this potential level of the 45 spikes were measured and plotted in the same way as the firing level in Fig. 12. It appears from the curve, Fig. 13, that this repolarization level varies from 70 to 61 mV and that for initial membrane potential values below 65—66 mV it represents an increased polarization, whereas for higher membrane potential values it represents a reduced polarization in relation to the initial membrane potential.

It has only been possible to perform experiments of this type on a limited number of interneurons and our material is too small to allow of general quantitative conclusions; the observations made suggest, however, that within certain limits both the firing level and the repolarization level are correlated to the level of the membrane potential and thus to the activity level of the neuron.

Spontaneous and naturally induced activity. Many interneurons have a spontaneous activity, as has been described by KOLMODIN and SKOGLUND (1954) and FRANK and FUORTES (1956). This activity may be increased or reduced by appropriate sensory stimulation and in such cases it is often possible to localize a peripheral source maintaining the discharges (cf. KOLMODIN 1957); in other cases peripheral stimulation does not influence the discharge rhythm which may then be maintained from other sources,

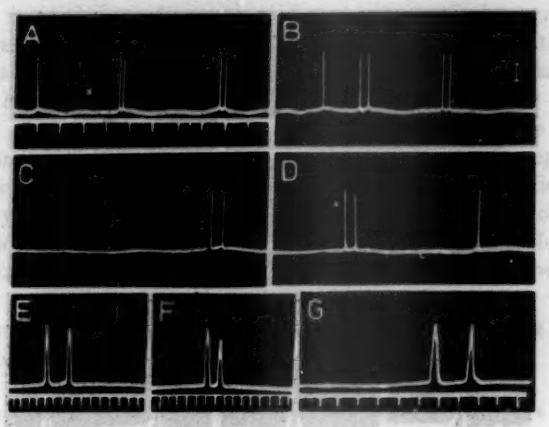

Fig. 14. Spontaneously discharging interneuron. *Time scale* in *A* 10 msec common for *A—D*, *E—G time* in msec. *Vertical bar* 10 mV.

e. g. from suprasegmental levels, or else may be assumed to be autogenous. Single shock stimulation of an afferent nerve may give rise to a diffuse increase of the spontaneous discharge frequency or to a burst of impulses often followed by a silent period of more than one second before the spontaneous activity starts again. Synchronized afferent volleys may also result in an immediate silencing of the cell.

Fig. 14 is from an interneuron belonging to the group of spontaneously discharging cells. From records *A—D*, selected from a recording on running film at rather slow speed, it is evident that fluctuations in the membrane potentials are continuously going on. On those of highest amplitude one or two spikes are elicited. It is apparent that in the double discharges the amplitude of the second spike is reduced at intervals below 2 msec (cf. records *E* and *F*) just as was the case in the artificially evoked discharges in Fig. 7. The prepotentials are of lower amplitude and are also building up more slowly than in the previous examples with electrical stimulation. It is obvious that such slow potentials may easily be missed when too short recordings are made (cf., *e. g.*, record *F*).

Fig. 14 exemplifies a spontaneous activity of rather low frequency, but many neurons have been observed to maintain a high

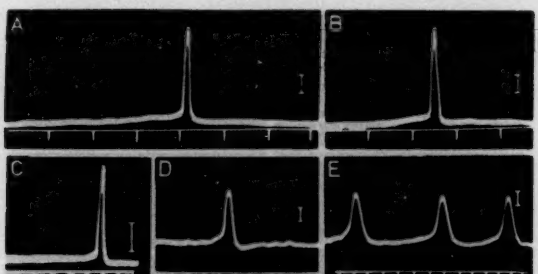

Fig. 15. Action potentials from interneurons during natural stimulation. *A*, *B* and *H*, muscle stretch; *C*, *D* and *E*, pad pressure; *F* and *G*, touch stimulation. *Time: A—B*, 5 msec; *C—H*, 1 msec. *Vertical bar* 10 mV.

irregular frequency for a considerable time; in one case a discharge of 200 per sec was followed for an hour.

Sustained rhythmical activity may be induced in initially silent neurons by natural activation of various peripheral sources (KOLMODIN and SKOGLUND 1954, FRANK and FUORTES 1956, SKOGLUND and KOLMODIN 1956). The characteristic frequency patterns induced by proprioceptive stimulation have been extensively analysed by KOLMODIN (1957).

In neurons discharging at a very slow rhythm one can observe slow potential fluctuations, similar to those in spontaneously discharging neurons, at the peaks of which spikes are fired (Fig. 15 *A—B*). As shown in a more detailed study of the slow potential changes during sustained activity of spinal neurons (KOLMODIN and SKOGLUND 1958), rhythmical discharges of higher frequency are associated with a steady depolarization. Each spike may then be preceded only by a small individual prepotential in the form of a deflection of short duration, as exemplified in records *C—E*. In other recordings (*F—H*) no distinct prepotentials at all were visible. In such recordings most of the spikes had a comparatively short duration of about 0.8 msec, with a range of values from 0.4

to 1.2 msec. Spikes of very short duration, as in *H*, have usually been recorded from units with less stable membrane potentials of lower amplitude than those from which spikes of medium duration (as in *F* and *G*) were obtained.

Out of the total number of experiments, including also those with artificial afferent stimulation, recordings without prepotentials represent about 50 per cent. According to the classification used by FRANK and FUORTES (1955), based on spike duration and absence of prepotentials, all spikes of this type should be derived from axons. Alternative explanations will be discussed below.

The post-spike events in naturally discharging neurons, which represent a complex process, will be dealt with in a following paper (KOLMODIN and SKOGLUND 1958).

Spike configurations. In the present series of experiments spike potentials showing "overshooting" have been observed only in about 10 per cent of the interneurons studied. The amplitude values of the action potentials, whether elicited by electrical or natural stimulation, have in the majority of the interneurons been 10—20 per cent lower than the membrane potential values. Spike potentials without an overshooting have been found in combination with both low and high membrane potential values; thus, *e. g.*, in interneurons with stable membrane potentials of 60—70 mV spike potentials of 40—50 mV have not seldom been observed.

As a rule no marked notch could be discerned on the rising phase of the spike; under certain circumstances, for instance when the membrane potential is deteriorating, such a notch may however appear. It has been observed at varying levels and should most likely be interpreted as a sign of a cell injury, indicating a delayed invasion of the structures involved.

Notches of this kind may also be observed in stages of relative refractoriness, as is illustrated in Fig. 16, showing an interneuron responding repetitively to electrical stimulation. In each such series of three spikes the ascending phase of the first spike shows no notch. This can also be seen in Fig. 16 *D*. Of these two curves the upper one is produced by a photoelectric function generator (cf. SUNSTEIN 1949) from a record belonging to the same experiment. The lower curve represents the first derivative of the upper potential (obtained by passing this potential through an amplifier of short time constant). The second and third spikes in the series display more or less pronounced notches, and simultaneously the

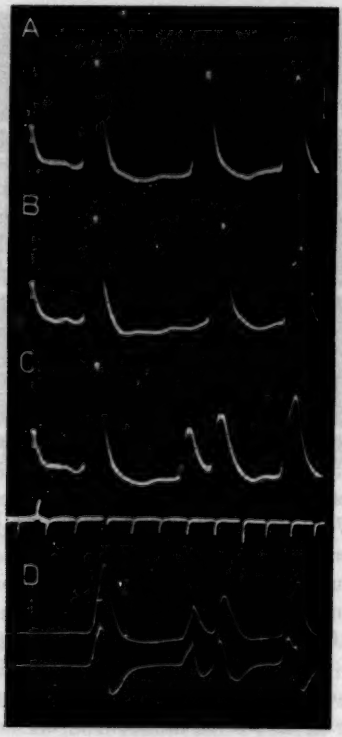

Fig. 16. Repetitive interneuron response to single shock stimulation of afferent nerve: *A*, *B*, *C* and upper curve in *D*. Lower curve in *D* represents first derivative of upper one, which is obtained by photoelectric scanning of paper mask of a record from the same series. *Time* in msec. *Vertical bar* 10 mV. See text.

duration of the spikes increases and their amplitude is reduced. The derivative of such a spike potential (lower curve, to the right, in *D*) shows that the rate of rise of the spike is reduced, as compared with the first spike, both in the phase below and that above the notch. As can be seen from the records the action potential may also be split up into two smaller "sub-spikes". These pictures suggest that the spike is built up of various fractions whose synchronization may be more or less perfect. We have, however, not made any experiments permitting us to judge of the closer nature and origin of these sub-fractions.

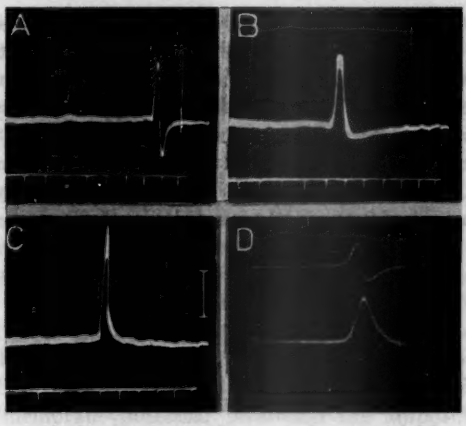

Fig. 17. Records taken during stepwise impalement of interneuron. *A*, diphasic spike obtained on approaching the cell; no d. c. shift. *B—C*, with membrane potential. *Upper curve* in *D* obtained by photoelectric wave form generator from *A*. *Lower curve* in *D* obtained from the upper one by integration. *A—C*, time in msec; *D*, between vertical lines 1 msec. *Vertical bar A—C* 10 mV.

A phenomenon observed on several occasions when the microelectrode is allowed to approach the cell very slowly is the recording of a positive-negative spike before any d. c. shift occurs. An example of such a spike potential showing its characteristic diphasic appearance is given in Fig. 17 *A*. A further movement of the electrode caused a resting potential to appear, and simultaneously the action potential changed into that shown in record *B*. A further minute movement caused a stepwise increase of both resting and spike potential which now became strictly monophasic (*C*).

As seen when comparing records *A* and *C*, the total duration of the diphasic potential is the same as that of the monophasic potential. This fact makes one suspect that the diphasic shape in *A* is actually due to a differentiation of a monophasic potential like that in *C*, by means of a very short time constant, *i. e.* a small capacitance coupling the action potential to the grid of the input tube. The capacitance could be either that across the wall of the micropipette with the tip resistance acting as the grid resistor of an ordinary a. c. coupled amplifier or, more likely, the capaci-

tance of the cell membrane, or parts of the membrane from previously penetrated cells, covering the tip of the electrode.

The inverse process of differentiation being integration it should be possible to restore the original shape of the potential by applying integration to record *A*. This has been done in record *D*. The upper curve corresponds to *A* and has been produced by means of a photoelectric function generator. This curve is then integrated with a time constant of 0.01 sec, the result being the lower curve.

Discussion.

Discharge types. It has been demonstrated that some interneurons may respond to single afferent volleys with a long-lasting discharge at high rate, whereas other neurons discharge with only one or two spikes in response to the same kind of stimulation, and all forms of transition types have been observed. For the present we abstain from an attempt to classify these neurons in various types on the basis of their response to electrical stimulation but the existence of such extreme differences in discharge pattern may suggest that the functional properties of various types of spinal interneurons are different, probably not only because of their anatomical arrangement but also because of their inherent membrane properties with regard to accommodation. The greater tendency to repetitiousness in interneurons as compared with motoneurons is of interest in view of the lower accommodation of peripheral sensory neurons as compared with motor axons (Skoglund 1942).

Slow potentials. In the present investigation we have, like Frank and Fuortes (1955), recorded spike potentials from interneurons preceded by prepotentials or lacking such. In agreement with these authors we are of the opinion that spikes accompanied by prepotentials should be considered as being recorded from soma. In different interneurons considerable variations have been observed in the amplitude and configuration of the prepotentials in response to artificial afferent stimulation. More or less favourable recording conditions may account for part of these variations but they are also likely to be indicative of differences in the density or the organization of the synapses within this very heterogeneous

group of neurons anatomically classified as belonging to the same category.

One factor determining the prepotential variations which may be observed in one and the same neuron is the level of physiological depolarization, as appeared from Fig. 12. This diagram shows that the firing level at which an impulse is set up by afferent single shock stimulation is not a constant level but is reduced when the membrane potential value is lowered by an increased afferent inflow; the firing level is, however, less reduced than the membrane potential level resulting in a decrease in prepotential amplitude with increasing depolarization of the cell. There is an interesting resemblance between these findings and observations (COOMBS, ECCLES and FATT 1955, ECCLES 1957) on motoneurons showing that there is an approximately linear relationship between the membrane potential level and the amplitude of the excitatory postsynaptic potential necessary for the initiation of a spike. However, it seems as if the firing level in these motoneurons is relatively constant and is not reduced to the same extent as in the present interneuron experiments. In the study of motoneurons the membrane potential was varied by means of currents applied through one barrel of a double-barrelled microelectrode, and in view of this and other differences in the experimental technique it is difficult to make a direct comparison with our results; thus the possibility cannot be excluded that the applied currents may influence the firing level.

The small transient fluctuations in membrane potential displayed by many interneurons in non-firing state may be autogenous or due to random synaptic bombardment from afferents or via adjacent units. The amplitude of such slow fluctuations can often be increased by sensory stimulation without any spikes being elicited. It is doubtful whether such spontaneous fluctuations are typical of all neurons; they have been missing in records from neurons in which according to all indications the recording has been made from soma. In such neurons sensory stimulation usually gives rise only to small, if any, slow fluctuations of the membrane potential. Differences of this kind may again be explained by varying recording conditions or cell injuries but may also reflect differences in membrane properties.

From a comparison between spike potentials recorded from motoneuron axons and somata, FRANK and FUORTES (1955) concluded that spike potentials of short duration *not preceded by*

slow potentials were recorded from axons and that this applied also to interneurons. Exact data on the dimensions of interneuron axons are lacking but they are more likely to be below than above 8—10 μ; as there is reason to believe that, with our present electrode technique, satisfactory recordings from fibres in the dorsal roots or the dorsal column with diameters below 10 μ can only exceptionally be made, it should be relatively difficult to obtain stable intracellular recordings from interneuron axons. With regard to spikes of very short duration, *viz.* about 0.3 msec, in which cases the membrane potential has usually been unstable and of low amplitude, we have not found any reason to doubt the interpretation proposed by FRANK and FUORTES.

Most of the spike potentials lacking prepotentials recorded in this investigation have, however, had a duration of about 0.8 msec which corresponds to the value of the duration of the spike potential in many interneurons with prepotentials, and in these units the membrane potentials have had a higher amplitude, a better stability and the recording has been longer-lasting than in units with spikes of very short duration. This might suggest that axons in the grey matter are far less easily injured than axons in the roots or else that these elements actually have greater dimensions than we have calculated with. There is also the possibility that spike potentials although not accompanied by prepotentials may still have been recorded from soma. The absence of a distinct prepotential may be due to the fact that the prepotential is liable to be overlooked, especially if it is slowly rising which is, in fact, the case for most of the naturally elicited impulses. Another possibility is that the neurons have initially been in a state of depolarization, in which case the amplitude of the prepotentials may be comparatively small (cf. above).

Spike configurations. In the majority of the interneuron recordings the amplitude of the action potential has been somewhat smaller than the value of the resting membrane potential, while only in a few cases "overshooting" has been observed. In the following some of the factors governing the relation between the measured action and resting membrane potentials will be briefly discussed.

Most of the pre-amplifiers used in connection with microelectrode recording for compensation of the high resistances and capacitive losses may give rise to both an over- and an undercompensation

of the frequency response (*viz.* the size of the spike potential); this is the case especially for such constructed according to the principle of a "dummy" electrode (cf. SOLMS, NASTUK and ALEXANDER 1953, FRANK and FUORTES 1955). An advantage of the preamplifier used in the present investigation (HAAPANEN and OTTOSON 1954) is that it can be adjusted without the approximation necessary when using other types. In the course of the experimental procedure repeated controls have been made of the adjustment for one and the same electrode. We therefore feel justified to rule out undercompensation as a reason why most of the spikes do not show any overshooting.

In the discussion of the first section an attempt has been made to estimate the influence on the recorded membrane potential of an injury caused to a restricted part of a cell membrane. Even if the idea of a simple potential division should be justified when applied to the resting potential, the much more complicated situation during activity may result in quite another potential division. Thus, *e. g.*, the capacitance of the membrane can no longer be neglected, as has been done when considering the value of the recorded membrane potential. The degree of injury caused to the mechanism underlying the resting potential may also be different from that caused to the action potential mechanism.

Different examples of a reduction of the amplitude of spikes appearing at short intervals have been given. This may be due to incomplete depolarization or partial invasion of the structures involved. If, as suggested by the records in Fig. 16, the spike is built up out of sub-fractions, their degree of synchronization apparently constitutes one possible factor determining the spike amplitude.

Considerable variations in the "afterpotentials" in different neurons have been observed; in some interneurons the spike repolarization level represents an increased, in others a decreased polarization as compared with the initial membrane potential level. Such variations from neuron to neuron may be due to specific membrane properties of various types of cells. However, also in one and the same neuron variations of this kind have been observed, and these could be related to physiological variations in the membrane potential level; at a low membrane potential value the spike is followed by a phase of increased polarization and at a high membrane potential value by a phase of reduced polarization as compared with the pre-spike level. These results are in agree-

ment with those obtained by KOLMODIN and SKOGLUND (1958) on motoneurons during naturally induced membrane potential variations. There is a close resemblance between these results on spinal neurons and the observations by EYZAGUIRRE and KUFFLER (1955) on the influence of natural polarization on afterpotentials of antidromic impulses in crustacean stretch receptor cells. Again, there is a certain discrepancy between these results and those obtained in studies on artificially polarized motoneurons, in which there occurs an afterhyperpolarization even at membrane potential values of 89 mV (COOMBS, ECCLES and FATT 1955).

Summary.

Intracellular recordings have been performed from interneurons in the lumbar segments of the spinal cord of non-anesthetized spinal cats. A full account is given of the experimental technique used in this and other investigations in the same series.

Section I. Continuous inkwriter recordings of the membrane potential level obtained from interneurons in various parts of the cord cross section are presented and compared with records from afferent fibres and motoneurons. Data of the average amplitude values and recording times for the different types of units are given.

Various factors influencing the values of the recorded membrane potential are discussed and an attempt has been made to find a relation between the degree of injury, cell size and recorded membrane potential.

Section II. The most common types of interneuron discharges in response to synchronous afferent volleys are described and typical variations in prepotential configuration are illustrated.

The firing level at which an impulse is set up by afferent single shock stimulation has been analysed in some detail in an experiment in which physiological variations in the membrane potential level were induced by natural sensory stimulation. It was found that the firing level is not constant but is reduced with lowering of the membrane potential value in an approximately linear way.

The spike repolarization level was also found to vary with the membrane potential level in a similar way which may result in

levels of increased or decreased polarization as compared with the pre-spike level.

Records of spontaneous and naturally induced activity are presented showing the relation between slow depolarization processes and spike discharges.

In experiments including both natural and artificial activation records have been obtained showing no distinct prepotentials. In such recordings the spikes have usually been of short duration (average 0.8 msec as compared with 1.3 msec for spikes accompanied by slow potentials). Some of these cases may represent axon recordings, but possible explanations of an absence of recordable prepotentials from soma are also discussed.

In the majority of interneuron recordings the spike amplitude has been 10—20 per cent lower than the membrane potential value; overshooting has only been observed in about 10 per cent of the recordings. Some factors influencing the spike amplitude have been taken up for discussion.

Special attention has been given to variations in spike configuration in the form of pronounced notches and a splitting up into sub-spikes; these variations have been interpreted as signs of a fractionated function of the cell membrane.

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Uber die Anzahl der motorischen Impulse (die Periodenzahl) des Elektromyogramms als quantitativer Ausdruck der voluntären, zentralen Kontrolle der Muskelaktion.

Von

R. M. BERGSTRÖM.

Bei der Redaktion am 6, Mai 1958, eingegangen.

Bei der Definierung des Begriffs der voluntären Kontrolle einer Muskelaktion gemäss den allgemeinen Prinzipien der Physiologie kann man die „Voluntärität" der Muskelaktion nicht auf das subjektive Erlebnis des Ausführers der Aktion zurückführen, da man das Erlebnis nicht mit physiologischen Mitteln messen kann. Deshalb identifizieren viele Forscher füglich den Begriff der Voluntärität mit der zentralen Kontrolle der Muskelaktion (SHERRINGTON 1947), welche Identifikation auf die Frequenz-Korrelation (ADRIAN und BRONK 1929) der Muskelaktionsströme und der entsprechenden Aktionsströme der Motoneurone bei der voluntären Muskeltätigkeit zurückgeführt wird. Das Zentralnervensystem kann die Muskeltätigkeit mit Hilfe der Anzahl der Motoneuronen, ihre Aktionsdichte (Frequenz) und ihrer Aktionsdauer regulieren.

Bei der Untersuchung der quantitativen Verhältnisse der voluntären Kontrolle einer Muskelaktion lässt sich vorteilhaft die sinnesphysiologische Untersuchungsmethode ausnutzen. Der Begriff

der voluntären Kontrolle wird hierbei auf die Fähigkeit zur Ausführung exakter, zielgesetzter Muskelaktion begründet, wobei die Genauigkeit und Steuerbarkeit der Aktion aus den Korrelationen zwischen den von der Muskeltätigkeit hervorgerufenen Erlebnissen und den mechanischen Leistungen des Muskels messbar sind. Man verfährt dabei gewöhnlich so, dass man die Versuchsperson erlebnismässig äquivalente Muskelaktionen ausführen lässt und dann in der mechanischen Tätigkeit des Muskels Äquivalenzen aufsucht, die mit der erlebnismässigen Äquivalenz korrelieren (Topologie). Die Fähigkeit des Versuchsperson zur Ausführung erlebnismässig gleichgrosser Aktionen ist gerade die Fähigkeit, exakte, gelenkte Muskelaktionen auszuführen, d. h. die Fähigkeit zur voluntären Kontrolle des Muskelaktionen. Was die organisch-physiologische Entsprechung dieses Kontrollierens ist, gehört nicht primär in den Objektkreis der Sinnesphysiologie, lässt sich aber mit ihr verbinden. So kann man z. B., unter Berücksichtigung der vorstehend angeführten physiologischen Definition der Voluntär-Kontrolle, annehmen, dass die Erlebnisäquivalenz bei den Voluntär-Aktionen von denjenigen Faktoren des Zentralnervensystems abhängig ist, mit deren Hilfe dieses die Muskelaktivität kontrolliert. Die propriozeptive Innervation des Muskelgewebes spricht jedoch auch zugunsten eines „peripheren" Faktors, der also als sensorischer Faktor neben dem vorstehend erwähnten motorischen oder „zentralen" Faktor die mit der Muskelaktion verbundene voluntäre Kontrolle ausmachte.

Zur Untersuchung dieses Vorgangs geben wir unserer Fragestellung eine für die sinnesphysiologische Methode geeignete Formulierung. Bei der Untersuchung der Korrelation zwischen Erlebnis und Reiz (bzw. in unserem Fall Muskelprozesse) in der vorliegenden Beobachtungssituation findet im Versuch Messen in zwei wesentlich verschiedenen Erscheinungskreisen statt. Einerseits misst der Ausführer der Aktion erlebnismässige Äquivalenzen, anderseits der Untersucher physikalische Äquivalenzen im Bereich der Muskelaktionen. Das erstere Messen geschieht im Kreis der Erlebnismannigfaltigkeit, das letztere im Kreis der Reizmannigfaltigkeit (Reenpää 1958), und die Relation zwischen diesen Kreisen lässt sich in Form des mathematischen Abbildungsverhältnisses

$$(1) \qquad Q \longrightarrow R$$

wiedergeben, wo Q den Kreis der Erlebnisse und R den Kreis der Begriffe, die den Reiz beschreiben, darstellt (Reenpää 1953).

Falls der Äquivalenz im Q-Kreis eine Äquivalenz im R-Kreis entspricht, ist der Reiz „richtig" beschrieben, und zwischen Erlebnis und Reiz besteht eine positive Korrelation. Die in dieser Weise aufgefundenen, Reize beschreibenden Begriffe (Energie, Impuls usw.) genügen allen den Forderungen, die an einen Stimulus als „Verursacher" des Erlebnisses zu stellen sind, aber gleichzeitig befreit uns diese Darstellungsweise von den Begrenzungen, die der kausalen Denkweise anhaften. Ein derartiger Fall, wo man im Kreis der Sinnesphysiologie bei kausaler Denkweise offenbar nicht weiter kommt, its gerade das Erlebnis der voluntären Muskelaktion: man denkt sich den Reiz, d. h. „die Ursache" des Erlebnisses, in die peripheren Muskelprozesse verlegt, aber anderseits ist das aktive Subjekt selbst „die Ursache" dieses Erlebnisses, infolge der Voluntär-Kontrolle.

Die bei der voluntären Muskelkontraktion auftretende Beobachtungssituation lässt sich nun in vorstehender Form (Ausdruck 1) ausdrücken, wobei Q die „gewollte" (erlebnismässige) Aktion beschreibt und R die mit physikalischen Mitteln messbare Tätigkeit des Muskels. Man hat feststellen können, dass sich das Erlebnis der Muskelkontraktion durch das Produkt der vom Muskel erzeugten physikalischen Kraft (k_m) und der Wirkungsdauer (Δt) dieser Kraft beschreiben lässt, d. h. durch den Begriff des vom Muskel entwickelten Kraftimpulses (I_m) (Renqvist 1930, Bergström 1957).

$$Q \longrightarrow k_m \cdot \Delta t \ (= I_m). \tag{2}$$

Demgemäss entspricht der gewollten, erlebnismässigen (voluntär kontrollierten) Äquivalenz die Kraftimpuls-Äquivalenz in der mechanischen Leistung des Muskels.

Anderseits hat man festgestellt, dass bei der voluntären Muskelkontraktion ein lineares Abhängigkeitsverhältnis zwischen der von dem Muskel erzeugten physikalischen Kraft und der Potentialfrequenz der Muskelaktionsströme besteht, welches Verhältnis sich beim kleinen m. interosseus dors. I (Jalavisto, Liukkonen, Reenpää und Wilska 1938) sowie auch durch Vergleich der integrierten, elektrischen Aktivität mit der Muskelkraft am grossen Muskel des Unterschenkel (Lippold 1952) nachweisen liess. Beide Untersuchungen betrafen die isometrische Kontraktion und wurden mit nicht-selektiven Elektroden ausgeführt.

Von unserem Standpunkt aus bedeutet dies, dass in dem, die voluntäre Muskelkontraktion beschreibenden Ausdruck (2) sich

das die Muskelfrakt beschreibende Glied k_m durch den Begriff der Aktionspotential-Frequenz (ν_τ) substituieren lässt, womit der Ausdruck (2) die Form

$$(3) \qquad Q \longrightarrow H_I \cdot \nu_\tau \cdot \Delta t$$

annimmt, wo H_I der Proportionalitätskoeffizient ist, dessen Wert vom Verhältnis k_m/ν_τ abhängt. Falls die Wirkungsdauer der Muskelkraft mit der der Aktionsströme gleichsetzbar ist, lässt sich das Produkt $\nu_\tau \cdot \Delta t$ als die Anzahl der Aktionspotentiale (der motorischen Impulse) interpretieren, da $\nu_\tau \cdot \Delta t = n_\tau$. Der Ausdruck (3) lässt sich dann in die Form

$$(4) \qquad Q \longrightarrow H_I \cdot n_\tau$$

schreiben, wo n_τ die Anzahl der Aktionspotentiale oder die Periodenzahl in der Zeit Δt ist. Demgemäss entspräche den voluntär kontrollierten, erlebnismässig äquivalenten Muskelkontraktionen die Äquivalenz der Aktionspotentialanzahl (der Periodenzahl), was das Problem der im Folgenden vorzulegenden, experimentellen Untersuchung bildet.

Obgleich die lineare Proportionalität zwischen Muskelkraft und Aktionspotentialfrequenz des Muskels in den referierten Untersuchungen bei isometrischer Kontraktion festgestellt worden ist, dürfte man sie auch bei nicht-isometrischer Kontraktion für gültig halten, bei der die Muskelverkürzung gering ist, d. h. bei leichten Aktionen.

Methodik. Die Versuche wurden am Abduktionsmuskel des Zeigefingers (m. interosseus dors. I) ausgeführt, in dem die zu dieser Aufgabe eingeübten Versuchspersonen zu erlebnismässig gleich grossen Aktionen veranlasst wurden, wobei die Stösse die Masse eines genau abgemessenen Pendels trafen. Bei Kenntnis der Eigenschaften des Pendels, der Grösse der Masse und der Amplitude der entstandenen Pendelbewegung liessen sich die Kraftimpulse berechnen (Eigenschaften des verwendeten Pendels sowie Methodik, siehe BERGSTRÖM 1957). Bei der Ausführung des Versuchs lag der Arm der Versuchsperson auf einer weichen Unterlage mit der Handfläche frei auf einer Stütze ruhend, in der der Zeigefinger in eine leichte Schiene fixiert war, deren Bewegungsachse mit der der Abduktionsbewegung des Fingers zusammenfiel. Diese Bewegung fand in horizontaler Ebene statt. Der Finger traf die Bewegungslose Masse des Pendels in Ruhelage aus einem Abstand von 0.5 mm zwischen Distalgelenk und Pendel. In Moment des Auftreffens betrug der Winkel zwischen Längsachse des Fingers und Schwingungsebene des Pendels 90°. Die Messung der Amplitude der Pendelbewegung erfolgte unmittelbar optisch. Die Aktionsströme des tätigen Muskels wurden mit nicht-selektiven Oberflächenelektroden (⌀5.0 mm am Muskel und 10.0 mm

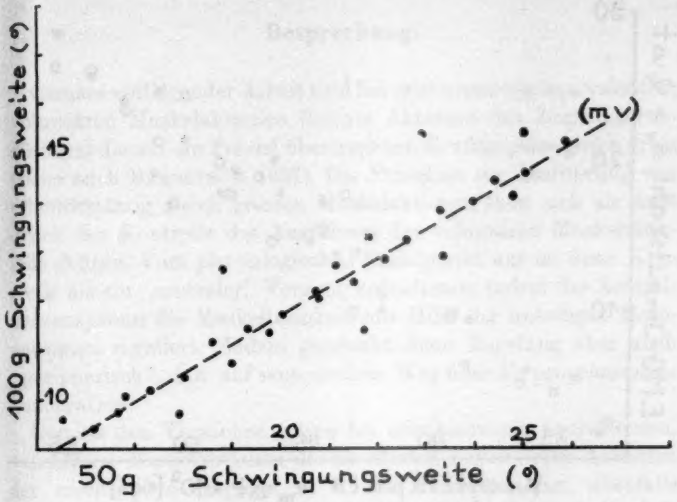

Fig. 1. Die Verteilung der erlebnisäquivalenten Pendelschwingungen bei der Zeigefingerabduktion. Pendelmassen 50 und 100 g.

am Ellbogen), mit Hilfe von Verstärker und Kathodenstrahloszillograph registriert. Die Berührungszeit zwischen Finger und Pendel wurde mit Hilfe von einem Elektronenschalter im EMG mitregistriert.

Die Versuche wurden mit 5 Versuchspersonen vorgenommen, die Pendelmassen betrugen 100 g und 50 g. Jede Versuchsperson führte erlebnisäquivalente Aktionspaare in der Weise aus, dass die Kraft der Stösse von Paar zu Paar variierte, während die Glieder der Stosspaare, das erste die Masse 100 und das zweite die Masse 50 g treffend, erlebnisäquivalent waren. Es wurden so 30 EMG-Registrierungen von erlebnisäquivalenten Stosspaaren erhalten, deren im Pendel hervorgerufenen Kraftimpulse bekannt waren. Die Berechnung der Anzahl der motorischen Impulse wurde nach dem gleichen Verfahren wie in der Arbeit von JALAVISTO u. Mitarb. (1938) ausgeführt. Da es sich um einen kleinen Muskel, nicht-selektiven Elektrodentechnik und schwache Aktionen handelt, kann man die aus dem Elektromyogramm berechneten Impulszahlen als proportional zu der Anzahl der im Muskel in Wirklichkeit auftretenden Impulse halten.

Versuchsergebnisse.

Figur 1 zeigt die von den erlebnismässig äquivalenten Stössen auf die Pendelmassen übertragenen Schwingungen, die Ordinate die Amplitude der 100 g Masse in Grad, die Abszisse die der 50 g

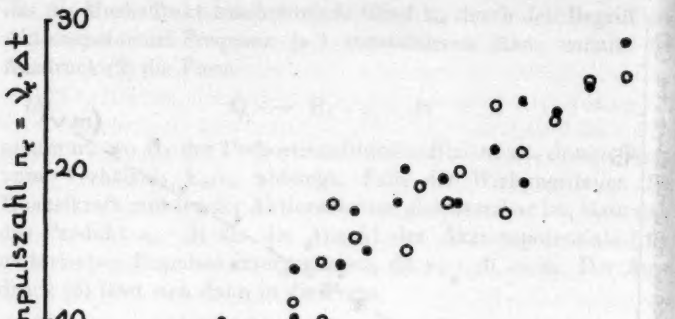

Fig. 2. Der Kraftimpuls der Zeigefingerabduktion und EMG-Impulszahl des m. interosseus dors. I bei erlebnismässig äquivalenten, voluntären Aktionen. Pendelmassen 50 (weisse Punkte) und 100 g (schwarze Punkte).

Masse. In das Koordinatensystem ist die theoretische Kraft-Impuls-Äquivalenz eingezeichnet (gestrichelte Linie mv), die die Schwingungsamplituden der kraftimpulsmässig äquivalenten Massen von 100 und 50 g bei verschieden kräftigen Stössen anzeigt. Aus dem Ergebnis ist ersichtlich, dass erlebnisäquivalente Stösse kraftimpulsäquivalente Schwingungen hervorrufen, in dem sich die einzelnen Versuchspaare angebenden Punkte um die theoretischen Linie mv gruppieren.

Von diesen Versuchspaaren, die in Figur 1 wiedergegeben sind, wurden 20 Versuchspaare ausgewählt, die am nächsten zur theoretischen Kraftimpuls-Linie lagen. Die diesen Werten entsprechenden EMG-Registrierungen sind in Figur 2 wiedergegeben. Die Ordinate gibt hier die aus den Registrierungen berechnete Anzahl der motorischen EMG-Impulse n_τ (Periodenzahl), die Abszisse den von der Aktion auf den Pendel übertragenen physikalischen Kraftimpuls an. Die Ergebnisse mit 100 g sind als schwarze, die mit 50 g als weisse Punkte eingetragen. Der mechanische, vom Muskel hervorgerufene Kraftimpuls der erlebnisäquivalenten Stösse steht in linearem Verhältnis zur Anzahl der aus dem EMG registrierten motorischen Impulse.

Besprechung.

Gemäss vorliegender Arbeit sind bei erlebnismässig äquivalenten, voluntären Muskelaktionen (leichte Aktionen des Zeigefingerabduktors) die auf ein Pendel übertragenen Kraftimpulse gleich gross (siehe auch BERGSTRÖM 1957). Die Fähigkeit zur Ausführung von erlebnismässig gleich grossen Muskelaktionen lässt sich als Ausdruck der Kontrolle des Ausführers der voluntären Muskeltätigkeit deuten. Vom physiologischen Standpunkt aus ist diese Kontrolle als ein „zentraler" Vorgang aufzufassen, indem das Zentralnervensystem die Muskeltätigkeit mit Hilfe der anteiligen Motoneuronen reguliert. Zudem geschieht diese Regelung aber auch „peripherisch", d. h. auf sensorischem Weg über die propriozeptive Innervation.

Gemäss den Versuchen waren bei erlebnismässig äquivalenten, voluntären Muskelaktionen die im Muskel registrierten Anzahlen der motorischen Impulse, d. h. die Periodenzahlen, ebenfalls äquivalent. Dies lässt sich so deuten, dass bei voluntären Muskelaktionen das Zentralnervensystem die Aktion mit Hilfe der Anzahl der motorischen Impulse kontrolliert, welche Anzahl somit der Ausdruck des Erlebnisses ist. Dies lässt sich, unter Anwendung der sinnesphysiologischen Darstellungsweise, durch den in der Fragestellung angeführten Ausdruck

$$Q \longrightarrow H_I \cdot n_\tau \tag{4}$$

beschreiben, wo Q die voluntäre Kontrolle der erlebnismässigen Muskelaktion bezeichnet, n_τ die Anzahl der im Muskel registrierten motorischen Impulse und H_I den Proportionalitätsfaktor, der vom Verhältnis k_m/ν_τ, d. h. zwischen entwickelter physikalischer Kraft des Muskels und Frequenz der Muskelaktionsströme abhängig ist.

Dieses Versuchsergebnis steht damit im Einklang, dass das Zentralnervensystem die Muskeltätigkeit im Bereich der Tätigkeit der Motoneuronen kontrolliert, denn in die Anzahl n_τ der motorischen Impulse gehen gerade die drei Faktoren ein, die als Formen der zentralen Kontrolle dargelegt wurden: 1) Anzahl der tätigen Motoneuronen, 2) deren Frequenz der elektrischen Aktivität und 3) Dauer dieser Frequenz. Die Anzahl der motorischen Impulse lässt sich als Produkt $n_\tau = \nu_\tau \cdot \Delta t$ aus Aktionsstrom-Frequenz und dessen Dauer ausdrücken, wobei die Frequenz ausser von der Frequenz des einzelnen Motoneurons auch von der Anzahl der

tätigen Motoneuronen abhängig ist. Somit gibt die Anzahl n_τ der motorischen Impulse die voluntäre, zentrale Kontrolle der Muskelaktion quantitativ wieder.

Es ist jedoch zu beachten, dass in unserem die Voluntärkontrolle beschreibendem Ausdruck (4) die Konstante H_I, hergeleitet aus der Proportionalität zwischen entwickelter Kraft des Muskels und Aktionsstrom-Frequenz des Muskels, von der Dimension des Kraftimpulses ist und sich in der Form

$$H_I = k_m \cdot \tau \left[\text{g. cm. sec}^{-1}\right] \tag{5}$$

(da $k_m = H_I \cdot \nu_\tau$ und $\nu_\tau = 1/\tau$) schreiben lässt, wo k_m die vom Muskel entwickelte Kraft und τ die Zeitperiode der Impuls-Frequenz bezeichnen. Somit beschreibt die Konstante H_I den vom tätigen Muskel hervorgerufenen Kraftimpuls während der Zeitperiode τ, und ist die Konstanz dieses Zeitperiode-Kraftimpulses ($k_m \cdot \tau$) das Mass der Konstanz der mechanischen Leistung des Muskels.

Ausdruck (4) lässt sich weiter in der Form

$$Q \longrightarrow n_\tau \cdot \left(k_m \cdot \tau\right) \tag{6}$$

schreiben. Er beschreibt die erlebnismässige, voluntäre Kontrolle der Muskelaktion quantitativ und enthält die an dieser Kontrolle teilnehmenden zwei Faktoren n_τ und $k_m \cdot \tau$, welcher letztgenannte bei unseren Versuchen die Konstante H_I ist. Die beiden Faktoren beschreiben nun verschiedene, an der voluntären Muskelaktion teilnehmende physiologische Prozesse. Der n_τ-Faktor, der die Anzahl der vom Zentralnervensystem dem tätigen Muskel zugeleiteten elektrischen Impulse oder der Zeitperioden bezeichnet, ist ein Mass für den zentralen Einfluss auf den physiologischen Zustand eines peripheren Organs, d. h. in unserem Fall des Muskels. Somit könnte man den n_τ-Faktor als einen „*zentralen Factor*" im obigen, die Voluntärkontrolle beschreibenden Ausdruck (6) ansehen.

Den Zeitperioden-Kraftimpuls $k_m \cdot \tau$ dürfte man als abhängig von den inneren Prozessen des Muskels erachten können, in dem er die Quantität der physikalischen Kraft angiebt, die im Muskel während einer Zeitperiode τ wirkt. Dieser Kraftimpuls, der oben als H_I-Faktor bezeichnet wurde, ist vom Verhältnis k_m/ν_τ abhängig, d. h. vom Verhältnis zwischen entwickelter physikalischer Kraft des Muskels und Frequenz der Muskelaktionsströme. Dieses Verhältnis variiert bekanntlich mit verschiedenen physiologischen Zuständen des Muskels. So ist z. B. ermittelt worden, dass bei

Müdigkeit und statischer Beanspruchung des Muskels (sog. Kohnstamm-Phänomen) sowie durch Temperaturwirkung sich das normale Verhältnis zwischen Muskelkraft und Aktionspotential-Frequenz verändert (JALAVISTO u. Mitarb. 1938, JALAVISTO, KERÄNEN und SEPPÄLÄ 1939, KNOWLTON, BENNETT und MCCLURE 1951 etc.). Diese Einflüsse, die sich also auf unseren H_I-Faktor beziehen, sind als periphere Einflüsse zu bezeichnen und sie werden von dem H_I-Faktor beschrieben. Diese peripheren Effekte kommen in dem die voluntäre Kontrolle beschreibenden Ausdruck (6) zum Vorschein, indem der H_I-Faktor variabel wird. Man könnte somit den H_I-Faktor im Ausdruck (4) als einen „*peripheren Faktor*" bezeichnen.

In den Ausdrücken (4) und (6) bezeichnet Q gemäss unserer sinnesphysiologischen Darstellungsweise die willentlich gesteuerte Aktion und ist als eine Art Quantitätserlebnis aufzufassen, das sich auf die ausgeführte Muskelaktion bezieht. Gemäss der kausalen Auffassungsweise ist dieses Erlebnis Q von dem Reiz $n_r \cdot H_I$ „verursacht" worden. Der Sachverhalt, dass dieser „Reiz" einen „zentralen" sowie einen „peripheren" Faktor enthält, steht damit im Einklang, dass das aktive Subjekt selbst „die Ursache" dieses Erlebnisses ist. Andererseits müssen wir uns aber denken, dass die Ursache des Erlebnisses in die peripheren Muskelprozessen verlegt ist. Die Notwendigkeit einer Reizbeschreibung, in welcher der Reiz sich nicht allein aus peripheren, d. h. Milieu-Faktoren zusammensetzt sondern auch einen subjektiven, d. h. vom observierenden Subjekt (Versuchsperson) abhängigen Faktor enthalten muss, ist von uns schon früher hervorgehoben worden (BERGSTRÖM 1957).

Rein organisch-physiologisch betrachtet eignen sich die Ausdrücke (4) und (6) gemäss unserer Fragestellung zur Beschreibung der voluntären bzw. zentralen Kontrolle der Muskelaktion, wobei Q diese Kontrolle angiebt. Es ist somit zu vermuten, dass das Zentralnervensystem bei voluntären Aktionen die Muskeltätigkeit steuert, indem es die Totalanzahl der vom motorischen Nervensystem dem Muskel zugeleiteten motorischen Impulse, Zeitperioden, reguliert. Jedoch vermag das Zentralnervensystem und die Voluntärkontrolle die mechanische Leistung des Muskels mit Hilfe der Anzahl der motorischen Impulse nur unter konstanten peripheren Verhältnissen zu regulieren.

Zusammenfassung.

In vorliegender Arbeit liess sich innerhalb der Grenzen der Versuchsbedingungen (leichte Muskelaktionen des m. interosseus dors. I) eine lineare Proportionalität zwischen Anzahl der im Muskel registrierten motorischen EMG-Impulse (Periodenzahl) und dem vom Muskel erzeugten physikalischen Kraftimpuls nachweisen. Da sich bei erlebnismässig äquivalenten Muskelkontraktionen eine Äquivalenz sowohl der Anzahl der motorischen Impulse als der vom Muskel erzeugten Kraftimpuls nachweisen liess, wird angenommen, dass die Kontrolle der voluntären Muskelaktionen teilweise im Bereich eines sog. „zentralen Faktors", d. h. durch Regulierung der Anzahl der motorischen Impulse im motorischen Nervensystem stattfindet, teilweise im Bereich eines sog. „peripheren Faktors", der den vom Muskel während einer Zeitperiode erzeugten physikalischen Kraftimpuls angiebt. Unter Berücksichtigung der Versuchsergebnisse wurde ein quantitativer Ausdruck hergeleitet, der diese voluntäre Kontrolle der Muskelaktion beschreibt und sowohl den zentralen als den peripheren Faktor enthält.

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A Rapid and Sensitive Method for the Determination of Total Serum Cholesterol.

By

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A sensitive method for total cholesterol determination has been published by HANEL and DAM (1955), based on the reaction of cholesterol with $ZnCl_2$ and acetyl chloride. This reaction (the Tschugaeff reaction) was originally employed by GÖRTZ (1934) for blood cholesterol determination. HANEL and DAM's method represents a modification and simplification of this original procedure. One advantage of the Tschugaeff reaction over the commonly employed Liebermann-Burchard reaction is its higher sensitivity, giving a 4-fold larger light absorption from a given amount of cholesterol. The colour is also relatively stable once formed. There is a slow decline in intensity with time, but not more than 3 per cent for 30 minutes at 20° C. This allows of simultaneous colour development in a series of tests, in contradistinction, for instance, to the Kingsley and Schaffert version of the Liebermann-Burchard test, where the colour must be developed individually for each tube, and the maximal density of the colour recorded.

The Kingsley and Schaffert method has, however, through its simplified extraction procedure, introduced a very valuable fea-

ture into the field of cholesterol determination. Chloroform is used to extract the free and esterified cholesterol from the serum, and following dehydration with $MgSO_4$, the colour is developed directly in an aliquot of this chloroform extract. This method also offers a convenient way of measuring ester cholesterol separately, through a rapid digitonin precipitation of the free sterol.

The present method has combined the extraction procedure of the latter with the colour reaction of the former method. It was found necessary, however, to modify both the extraction step and the colour development to some extent, in order to achieve accuracy in the determination.

Method.

Reagents.

KOH, 10 per cent aqueous solution
Ethanol-acetone, 1 : 1
Chloroform, anhydrous
$MgSO_4$, anhydrous
Acetyl chloride

$ZnCl_2$-reagent: Dissolve 120 g granular anhydrous $ZnCl_2$ in 359 ml glacial acetic acid. This is achieved by heating the suspension at 80° C with occasional stirring for 2—3 hours. After cooling to room temperature, filter the reagent through a sintered glass filter, and store it in a glass stoppered dark bottle. It may be used until it, due to absorption of moisture, no longer mixes with chloroform without turbidity. This deterioration may take more than 3 months to develop.

Cholesterol standard: Dissolve 100 mg cholesterol in 100 ml chloroform as a stock solution. Dilute this solution 1 : 20 for the working standard.

Procedure. Pipette 50 μl of serum into 15—25 ml glass stoppered centrifuge tubes. Add 1 ml of ethanol-acetone and 1 drop of 10 per cent KOH, and heat the tubes unstoppered on a 85° C waterbath for 10 min. After cooling to room temperature, 5 ml of chloroform and about 200 mg of $MgSO_4$ are added. The latter should recently have been heated over night at 150—200° C, and subsequently kept protected from moisture. The tubes are now stoppered and vigorously shaken on a Microid Flask Shaker, or other suitable shaking device, for 3 min. After centrifugal sedimentation of the solids, 2 ml aliqots are drawn from the supernatants and placed in 5 ml wide-neck volumetric flasks. 2 ml of the cholesterol standard is also measured out at this time, and 2 ml of chloroform alone for the reagent blank. To each flask of this series is now added 1 ml of the $ZnCl_2$-reagent and 1 ml of acetyl chloride. The contents are well mixed, and the flasks, loosely stoppered, put for 18 min in a 55° C water bath. At the end of this period, the flasks are rapidly cooled by immersion in ice-water. After dilution to the 5 ml mark with chloroform, the optical density (*OD*) is read in a colorimeter or spectrophotometer at 521 mμ against the blank.

If the *OD*-reading is A for a serum, and B for the standard cholesterol, then the serum cholesterol value C in mg % is given by

$$C = \frac{A \times 500}{B}$$

For serum cholesterol values between 500 and 1,000 mg%, as encountered in hypercholesterolemic cases, it is preferable to draw an aliqot of 1 ml instead of 2 ml from the lipid extract. The factor to be used in the above equation will then be 1,000. In two models of Coleman Junior Spectrophotometer 6A, the value of B was regularly about 0.40 and 0.51 respectively, with 12 × 75 mm tubes.

Discussion.

Procedure. A step of alkaline ethanol-acetone treatment has been included in the method, since, in our hands, some material would otherwise stick to the glass walls and remain partly unextracted. This was especially noticeable with rat sera. With the ethanol-acetone treatment included, serum values were found which agreed with those found after conventional and exhaustive saponification of the same sera. It is not necessary to neutralize the KOH afterwards. Whatever traces of KOH pass into the dehydrated chloroform phase has been shown not to affect the colour reaction measurably. The question whether the saponification of the cholesterol esters in the above treatment is complete or not, is not crucial, as the molar extinction values for cholesterol and cholesterol esters are the same (JÖRGENSEN and DAM 1957).

Development of the colour for 18 min at 55° C instead of 15 min at 65° C was found to give more reproducible results. The evaporation or boiling of the mixture is here less vigorous, and does not result in losses from overboiling. The intensity of the colour was read at 521 mμ, where the maximum was found, both for standard cholesterol (Merck, M. P. 147° C), and for the chloroform extract of serum (Fig. 1).

It is advisable to use a cholesterol standard for each set of determinations. Although it usually, within experimental errors, gives rise to the same *OD*, small variations in the purity of the reagents, the temperature of the water bath, and the effective heating time may introduce errors that largely will be compensated for when a standard is used.

Specificity. A number of sterols were tested by HANEL and DAM for possible interference in the determination. Of those tested, only

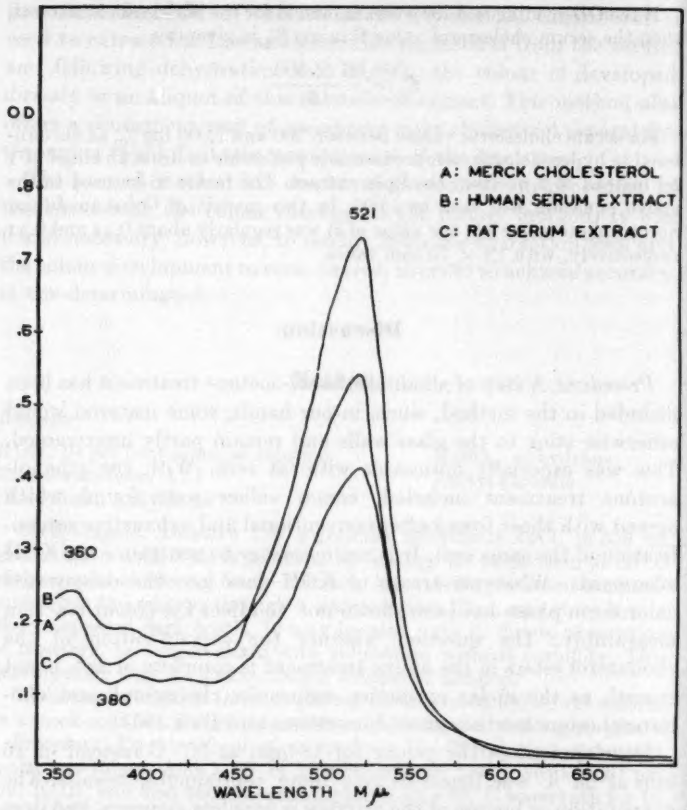

Fig. 1. Absorption spectrum of the cholesterol colour, recorded with a BECKMAN DK spectrophotometer. Chromatography of the Merck cholesterol on alumina, and subsequent recrystallization from ethanol yielded a product with a spectrum after cloour development identical with curve A.

coprostanol and stigmasetrol produced colour at 528 mμ, the former with an intensity of one tenth of that of cholesterol, the latter with an intensity somewhat in excess of the cholesterol value.

Accuracy. Series of 12 determinations on the same sera and on cholesterol directly were carried out. The colour intensity was measured in a Coleman Junior spectrophotometer in 12 × 75 mm tubes. The errors listed are those of the total procedure, included those of the apparatus.

At the level of 500 mg % serum cholesterol, which gives an *OD* of about 0.40, the standard deviation in the *OD* was found to be approximately 0.008 *OD* units, or ± 2 %. At the level of 70 mg %, giving an *OD* of 0.056, the standard deviation was ± 4.5 %. Approximately one half of this uncertainty was introduced at the extraction level, the remainder in the colour development and the reading of the colour, as shown when cholesterol in the corresponding amounts was assayed directly.

One hundred μg of cholesterol was regularly used as standard, giving an *OD* of 0.40. When computing the serum cholesterol values, the 1 % uncertainty in the standard *OD* contributes in the denominator of the expression, and the 2 to 4.5 % uncertainties in the numerator. With serum determinations in duplicate and the standard in triplicate, these relative uncertainties are reduced and compounded to the following ones for the average cholesterol values:

$$\sqrt{\left(\frac{4.5}{\sqrt{2}}\right)^2 + \left(\frac{1}{\sqrt{3}}\right)^2} = \pm 3.2\ \%, \quad \sqrt{\left(\frac{2.0}{\sqrt{2}}\right)^2 + \left(\frac{1}{\sqrt{3}}\right)^2} = \pm 1.5\ \%$$

The 70 mg % serum value thus has an expected uncertainty of ± 2 mg %, and the 500 mg % serum value an uncertainty of ± 8 mg %. Intermediate serum cholesterol values will have error limits between these.

In addition to these random errors from the inaccuracy of the various steps, one may expect a certain systematic error, due to colour produced by materials other than cholesterol. Inspection of Fig. 1 reveals an interference that increases in magnitude with decreasing wavelength. In order to assess the magnitude of this overestimation, the *OD*-values for pure cholesterol was obtained at 501 and 541 mμ, as well as at 521 mμ. If the ratios of the former *OD*'s to the *OD* at the maximum is designated f_1 and f_2, and the corresponding ratios for a serum cholesterol determination r_1 and r_2, then the ratio of the corrected to the observed *OD* at 521 is obtained according to the following expression:

$$\frac{OD_C}{OD_O} = \frac{2 - (r_1 + r_2)}{2 - (f_1 + f_2)}$$

This follows from simple geometrical considerations, on the assumption that the interfering colour changes linearly in the region 501—541. When this formula was applied to a series of 10 normal human serum cholesterol determinations, OD_c/OD_o

ratios of 0.93—1.00 were found, with 0.975 as the average. It should thus be borne in mind that routine determinations at a single wavelength overestimate the cholesterol content with the order of magnitude indicated.

Without such a correction for irrelevant absorption, the average value obtained in a series of 20 human sera with the present method was 6 % higher than that found in the same sera in a different laboratory, where a slightly modified Kingsley and Schaffert method was employed.

Summary.

Total cholesterol is determined in 50 μl of serum by extraction with chloroform. The acetyl chloride-$ZnCl_2$ colour reaction for cholesterol is carried out directly in this extract. The procedure includes an initial brief saponification step, and dehydration of the chloroform phase with anhydrous $MgSO_4$. Serum values for duplicate determinations are obtained with an accuracy of $\pm$ 2 % for intermediate cholesterol concentrations.

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Pulmonary Ventilation and Effect of Oxygen Breathing in Heavy Exercise.

By

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As is well known pulmonary ventilation increases relatively more than metabolism during heavy muscular exercise so that the ventilation per litre oxygen uptake is increased and the alveolar pCO_2 is diminished as compared to the corresponding values in light work. The reason for this hyperventilation was studied by ASMUSSEN and NIELSEN (1946). These authors found that breathing of air mixtures enriched with oxygen lowered the ventilation in heavy work but had no such effect in rest and light work. The arterial oxygen saturation was practically the same in heavy as in light work and it was therefore concluded that the hyperventilation could not be due to a lowered arterial oxygen tension. Nor could the hyperventilation be explained by an increased acidity of the arterial blood. Though it often has been found that the arterial pH decreases in exhausting work, in heavy work with highly trained subjects hyperventilation and the resulting hypocapnea may overcompensate the formation of lactic acid so that the arterial pH becomes normal or even increases. The hyperventilation, however, was found to be closely correlated to the degree of anaerobicity in the working muscles as judged by the blood lactate concentration. These experimental results seemed to be best explained by assuming that in muscles working under partly anaerobic

conditions a substance is produced which effects an increase in ventilation. In special experiments it was shown that the active substance could not be lactic acid itself.

This hypothesis was opposed by several authors, especially by BANNISTER and CUNNINGHAM (1954) who presented reasons for regarding the decrease in ventilation caused by breathing high oxygen during heavy work as being due to the abolition of an existing arterial hypoxemia. This view was also supported by ÅSTRAND (1956) who on the other hand admitted that in addition to the effect of such an arterial hypoxemia attention must be paid to the local metabolism in the working muscles.

In the present experiments the regulation of respiration in heavy work has been re-examined by studying the arterial oxygen tension and various respiratory functions in rest and work when breathing atmospheric air and when breathing air mixtures with high and low oxygen concentrations.

Methods.

The experiments were performed on a group (17 in all) of young normal male students. Each subject was studied several times but, because of the necessary arterial punctures, at intervals of about one week. The work was performed on KROGH's bicycle ergometer furnished with a reclining chair instead of the saddle. Ventilation and respiratory metabolism were determined by the DOUGLAS bag method in the steady state of work. The air analyses were performed on the SCHOLANDER apparatus. The arterial CO_2 and O_2 tensions were determined by a modification of KROGH's bubble equilibration method, details of which are described elsewhere (ASMUSSEN and NIELSEN 1958). In the low oxygen experiments the arterial pO_2 was determined from the oxygen saturation and the pH of the arterial blood, and the arterial pCO_2 and the arterial pH were determined as described by ASMUSSEN and NIELSEN (1956). Alveolar gas tensions during work were calculated from the BOHR formula using the average values of the physiological dead space determined by ASMUSSEN and NIELSEN (1956). In the rest experiments direct samples of end-tidal air were collected by the automatic device described by NIELSEN and SMITH (1951).

The experiments were performed in the morning, two to three hours after the subjects had had a light breakfast. (In the rest experiments and in the experiments with light work the subjects were fasting.) The arterial puncture was performed 4 to 5 minutes previous to the determination of ventilation and metabolism, and in the middle of this period the blood was drawn through an indwelling arterial needle into a tube kept in ice and water and containing a drop of heparin under paraffin oil. When end tidal air was sampled the time of collection coincided with

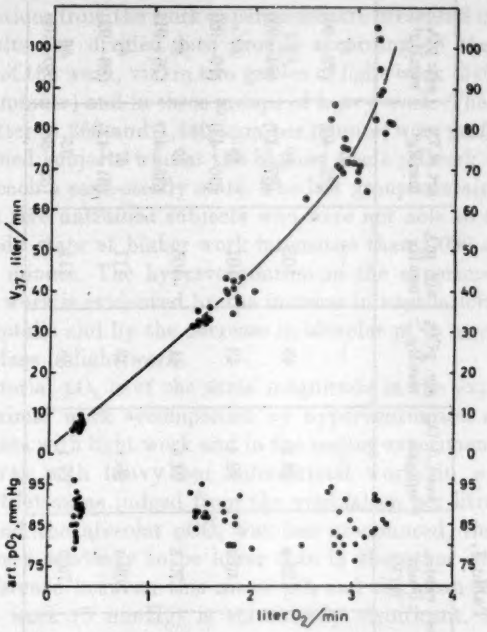

Fig. 1. Pulmonary ventilation (BTPS) and arterial oxygen tension in relation to oxygen consumption from experiments on 11 male subjects.

the time of blood drawing. The drawn arterial blood (12 to 20 ml) was gently mixed with the heparin and immediately transferred anaerobically to the cooled equilibration syringes. The blood was stored on ice and rewarmed to 37° C in water just before the introduction of the air bubble. The results were corrected corresponding to differences between rectal temperature during the work and temperature at equilibration of the bubble.

Results.

Fig. 1 shows the individual determinations of pulmonary ventilation and of arterial pO_2 in relation to oxygen uptake. Each point represents the mean of two single determinations. In the experiments with the heaviest work a pulmonary hyperventilation occurs, which can be seen by the increased steepness of the ventilation curve in this range. The average values of these and other respira-

Table I.

	Subjects	number of determinations	O_2-uptake l/min. STPD	vent. l/min. BTPS	vent./l O_2-uptake	alv. pCO_2 mmHg	alv. pO_2 mmHg	art. pCO_2 mmHg[1]	art. pO_2 mmHg[1]
Resting	7	18	—	—	—	—	—	*37* (32—40)	*87* (79—96)
Light work	6	10	1.40	33.2	22.2	43	103	*40* (37—43)	*88.5* (84—95)
"	6	10	1.81	40.4	22.3	43	101	*39* (36—43)	*87* (79—92)
Submaximal work .	4 trained subjects	9	2.05	70.8	24.0	41	105	*39* (35—43)	*82* (76—91)
Maximal work	4 trained subjects	9	3.30	88.5	26.8	39	111	*37* (31—42)	*87* (83—92)
"	5 untrained subjects	6	2.79	75.5	27.1	38	111	*35* (32—38)	*86* (80—94)

[1] Figures in parentheses denote range.

tory functions from the work experiments are presented in Table I. The results are divided into groups according to the relative severity of the work, viz. in two grades of light work (540 and 720 kgm per minute) and in three groups of heavy work. The first two of the latter (1,260 and 1,440 kgm per minute) were performed on four trained subjects who at the highest grade of work were just able to reach a semi-steady state. The last group contains experiments on five untrained subjects who were not able to reach the semi-steady state at higher work intensities than 1,080 and 1,260 kgm per minute. The hyperventilation in the experiments with maximal work is evidenced by the increase in ventilation per litre oxygen uptake and by the decrease in alveolar pCO_2 as compared to the values in light work.

The arterial pO_2 is of the same magnitude in the experiments with maximal work accompanied by hyperventilation as in the experiments with light work and in the resting experiments. In the experiments with heavy but submaximal work, in which the hyperventilation as judged from the ventilation per litre oxygen uptake and the alveolar pCO_2 was less pronounced, the arterial pO_2 shows a tendency to be lower than in the other conditions. (The difference between this mean pO_2 and the mean pO_2 at the maximal work (5 mmHg) is statistically significant, the ratio $\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\sigma_1^2 + \sigma_2^2}}$ being 2,5.)

When oxygen is added to the inspired air during heavy work the ventilation is lowered. This can be seen from the experiments in Fig. 2 which shows the ventilation and the alveolar pCO_2 during work at an intensity of 1,440 kgm per minute when the inspired air was atmospheric air or contained 33 per cent oxygen or 99 per cent oxygen. The results from 12 corresponding series of experiments on 7 subjects are presented in Table II, which shows that the breathing of 33 per cent oxygen lowers the ventilation and raises the alveolar pCO_2 and that breathing of 99 per cent oxygen causes a further decrease in ventilation and increase in alveolar pCO_2. This graded effect of adding oxygen to the inspired air was evident in the experiments with all the subjects. Table II also contains the average values for arterial pO_2 from parallel series of experiments, 15 with heavy work while breathing air, 9 while breathing 33 per cent oxygen and 9 while breathing 99 per cent oxygen. In these experiments which were performed partly on the ame subjects as those mentioned in the upper part of the table,

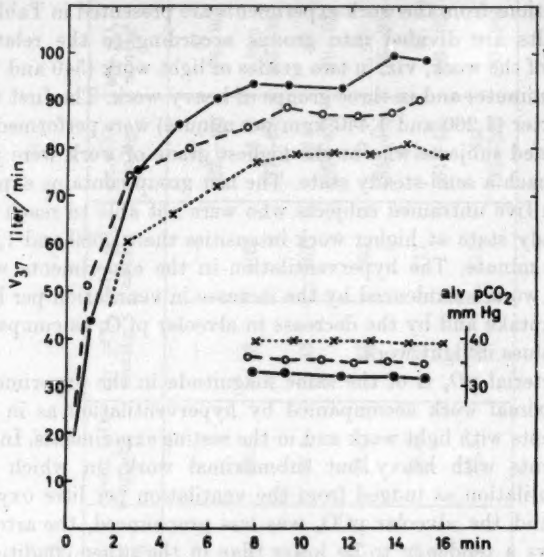

Fig. 2. Pulmonary ventilation (BTPS) and alveolar CO_2 tension during work (oxygen consumption 3.1 litres per minute) while breathing air ●——●, 33 per cent oxygen ○——○ and 99 per cent oxygen ×——×.

the average arterial oxygen tensions were 87 mmHg, 183 mmHg and 663 mmHg, respectively.

In light work (540 kgm per minute) it was found in 5 series of experiments on 4 subjects that the breathing of 99 per cent oxygen had no effect on ventilation and on alveolar pCO_2 (V_{37} = 33.2 l/min and 33.7 l/min; alv. pCO_2 42 mmHg and 43 mmHg in the pure oxygen and the air experiments, respectively.

Discussion.

The present experiments show that the hyperventilation which occurs in heavy work and which can be abolished or reduced by breathing oxygen enriched mixtures cannot simply be due to a lowered arterial pO_2, since this was found to be the same in maximal work as in light work and rest. Earlier determinations of the

Table II.

inspired air:		atm. air			33 per cent O_2			99 per cent O_2	
subj.	number of experiments[1]	O_2-uptake l/min. STPD	ventilation l/min. BTPS	alv. pCO_2 mmHg	O_2-uptake l/min. STPD	ventilation l/min. BTPS	alv. pCO_2 mmHg	ventilation l/min. BTPS	alv. pCO_2 mmHg
G. G. R.	2	2.55	66.2	37	2.50	62.9	39	55.5	44
P. J. V.	2	3.25	83.1	38	3.15	73.1	42	65.7	46
J. M.	2	2.95	71.1	39	2.90	63.8	43	57.6	46
P. G.	1	3.03	90.1	35	2.90	73.1	40	69.5	42
H. E. C.	1	3.50	104.5	35	3.49	88.5	39	81.0	42
J. S. L.	2	3.12	95.6	32	3.07	83.1	37	74.7	41
F. J.	2	2.75	87.7	32	2.63	77.8	34	69.3	38
average:		3.02	*85.5*	35.4	2.95	*74.6*	39.1	*67.6*	42.7
art. pO_2 average and range			*87* (80—94) n = 15			*183* (161—190) n = 9		*663* (635—685) n = 9	

[1] In each experiment 4 determinations of ventilation and alv. pCO_2 were made during the steady state of work (8.—15. min.).

arterial pO_2 during exercise were mostly performed during light work. A table containing a comparison of earlier results obtained both at rest and during light exercise is given by BARTELS *et al.* (1955). The average rest values varied from 85 to 94 mmHg and the exercise values from 83 to 92 mmHg. LILIENTHAL *et al.* (1946) besides the experiments with light work, in which the average arterial pO_2 was found to be 89 mmHg, also made three experiments with heavy work (oxygen consumption about 3 l/min) and found an arterial pO_2 of 72 to 74 mmHg. In the present experiments the average arterial pO_2 both at rest and during light and heavy work was found to be from 82 to 89 mmHg, and the values during maximal work were the same as in rest *i. e.* about 87 mmHg (see Table I).

This value is clearly below the threshold value of the arterial chemoreceptors as found in animal experiments by WITZLEB *et al.* (1955). These authors found the threshold value to be about 115 mmHg and further that the greatest sensitivity to changes in the arterial pO_2 was in the range just below this value. This last observation is at variance with the results of EULER, LILJESTRAND and ZOTTERMAN (1939), which according to the sensitivity curve as calculated by WITZLEB *et al.* shows the lowest sensitivity at the pO_2 values close to threshold. Also the results of ÅSTRAND (1954) on cats partly acclimatized to high altitude seem to show a progressively increasing impulse frequency in the chemoreceptor nerves at decreasing oxygen tension of the inspired air.

In agreement with the observation that the arterial pO_2 in normal conditions is below the threshold values of the chemoreceptors are the results of experiments on humans in which the inspired air suddenly has been changed from room air to oxygen rich gas mixtures. DRIPPS and COMROE (1947), LOESCHCKE (1953) and DEJOURS *et al.* (1957 a) found in such experiments during rest a small immediate decrease in pulmonary ventilation and DEJOURS *et al.* (1957 b) who studied the effect of one single breath of oxygen, found during light to moderate exercise a percentage decrease in ventilation of about the same size as in the rest experiments. The immediate decrease in ventilation on sudden inhalation of oxygen rich gas mixtures was in the rest experiments of DRIPPS and COMROE and of LOESCHCKE followed by a secondary rise up to or above the normal resting ventilation in spite of continued oxygen breathing. A definite explanation of this secondary rise in ventilation has so far not been given but the above authors discuss several

possible reasons for it. Also in steady state experiments with light work (ventilation less than about 20 litres per min) oxygen breathing produces a slightly elevated ventilation (ASMUSSEN and NIELSEN 1946). During the steady state of heavy exercise, however, the latter authors found a rapid and large decrease in ventilation on changing from breathing room air to breathing pure oxygen, and the ventilation continued to be low as long as the oxygen breathing was maintained. On changing back to room air breathing the ventilation rapidly returned to the same level as before oxygen breathing.

On the basis of the above mentioned animal experiments which show that the here presented values of the arterial pO_2 (Table I) both in rest and exercise are low enough to stimulate the chemoreceptors, and in accordance with the sudden decrease in ventilation found in the experiments on humans, it is possible partly to explain the decrease in pulmonary ventilation on oxygen breathing in heavy exercise as the result of an abolition of a hypoxic chemoreceptor drive, if one assumes that the chemoreceptor impulses increase the sensitivity of the respiratory centre towards the work stimulus. This assumption is supported by the above mentioned observation of DEJOURS *et al.* that the percentage decrease in ventilation following one breath of pure oxygen is the same in rest and work, although it must be born in mind, that the inspired amount of oxygen must have been larger during work due to the larger tidal volume. Also work experiments in low oxygen lend support to the assumption that the chemoreceptive drive sensitizes the respiratory centre to the work stimulus. As seen in Fig. 3 and as shown earlier by CHRISTENSEN (1937), HICKHAM *et al.* (1951), and ÅSTRAND (1954) the pulmonary ventilation with increasing oxygen uptake increases at a much steeper rate in low oxygen than in normal air. If this steeper increase in ventilation during work in low oxygen is due to a hypoxic chemoreceptive stimulus the effect of this stimulus must be a sensitizing one because the arterial pO_2 (Fig. 3 b) and, therefore, probably the frequency of the impulses from the chemoreceptors has been nearly the same at the different intensities of work. It is possible, though, that the steeper increase in ventilation is influenced also by the high degree of anaerobic metabolism which has existed in the low oxygen experiments (see Fig. 3 b). The anaerobic factor cannot have been an increased acidity of the blood as the arterial pH in the low oxygen experiments was equal to or higher than the arterial pH in the corresponding experiments in normal air (Fig. 3 b).

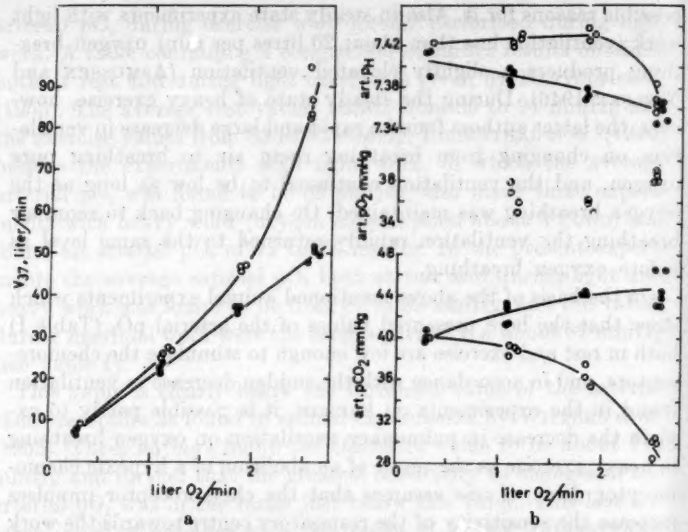

Fig. 3 a. Pulmonary ventilation (BTPS) in relation to oxygen consumption while breathing air ●——● and while breathing 12.6 per cent oxygen ○——○.

Fig. 3. b. Arterial pH, arterial oxygen tension and arterial CO_2 tension in relation to oxygen uptake while breathing air ●——● and while breathing 12.6 per cent oxygen ○——○. Data from same experiments as in Fig. 3 a.

A sensitizing effect on the respiratory centre of chemoreceptor impulses as that suggested here to the work stimulus has previously been demonstrated also to the CO_2 stimulus for pCO_2 values above threshold by NIELSEN and SMITH (1951). CUNNINGHAM and O' RIORDAN (1957) have demonstrated an analogous effect on the centre of an increased body temperature to the CO_2 stimulus. The combined effect of CO_2 and the work stimulus, however, rather seems to be simply an additive one (NIELSEN 1936, ASMUSSEN and NIELSEN 1957).

Even with the assumption of an increased sensitivity of the respiratory centre to the work stimulus caused by the hypoxic stimulus is it difficult completely to explain the hyperventilation of heavy exercise and its abolition through oxygen breathing as the result of an existing chemoreceptive drive and its disappearance when the arterial oxygen tension is increased. As seen in Table II and in Fig. 2 the full effect of increasing the oxygen pressure of the inspired air on pulmonary ventilation is not reached with 33 per

cent oxygen in spite of the fact that the arterial oxygen tension reaches the high value of 183 mmHg. This value is much higher than the threshold value of the chemoreceptors which in the animal experiments of WITZLEB *et al.* (1955) was found to be about 115 mmHg. Correspondingly DEJOURS *et al.* (1957 a and b) from their experiments in rest and work on humans concluded that the chemoreceptive drive was abolished when the oxygen percentage in the inspired air was above 33 per cent. Unless the threshold value for the chemoreceptors lies at a considerably higher oxygen tension than indicated by the above mentioned experiments the further decrease in pulmonary ventilation found in heavy work when going from 33 per cent to 99 per cent oxygen in the inspired air (see Table II and Fig. 2) must be caused by some other mechanism. There is at present no reason for assuming that the threshold of the chemoreceptors is changed during heavy work as compared to rest or light work.

In earlier studies (ASMUSSEN and NIELSEN 1946) it has been found that there is a close correlation between the hyperventilation in work and the degree of anaerobiosis in the working muscles (*e. g.* in heavy work with the legs, at more moderate work when this is performed with the arms and in work during moderate CO poisoning). Further it was found that the hyperventilation was diminished or eliminated by oxygen breathing at the same time as the anaerobiosis as judged from the blood lactates was diminished. It was therefore suggested that during anaerobic work a substance was produced which had the effect of increasing the ventilation. During oxygen breathing it was supposed that the increased amount of physically dissolved oxygen made the work less anaerobic so that the decrease in ventilation could be explained at least partly in this way. It is possible that the decrease in ventilation that occurs when pure oxygen is substituted for 33 per cent oxygen in the inspired air and which is difficult to explain by the assumption of an abolished chemoreceptive drive is caused by such a diminished anaerobicity in the working muscles, as the physically dissolved oxygen increases considerably when the arterial oxygen tension increases from 183 to 663 mmHg (see Table II). As shown earlier (ASMUSSEN and NIELSEN 1946) the active substance cannot be the lactic acid itself because the changes in ventilation is completed much more rapidly than the changes in blood lactate level both when switching from air breathing to oxygen breathing and when switching back again from oxygen breathing to air breathing.

Accordingly the hyperventilation during heavy work which can be diminished or eliminated by oxygen breathing seems to be explainable partly as the result of chemoreceptive impulses which sensitize the respiratory centre towards the work stimulus and partly to the anaerobic production of some substance which acts to increase ventilation. Oxygen breathing then diminishes ventilation in heavy work *1.* by abolishing the chemoreceptive drive and *2.* by making conditions in the working muscles more aerobic.

Summary.

1. The regulation of respiration in heavy work has been studied in young normal subjects by measuring the arterial oxygen tensions and various respiratory functions in rest and work when breathing atmospheric air and when breathing air mixtures with augmented or lowered oxygen concentrations.

2. It was found that the arterial pO_2 in heavy work with pronounced hyperventilation (*i. e.* increased ventilation per litre oxygen uptake and decreased alveolar pCO_2) was of the same magnitude (87 mmHg, range 82—94 mmHg) as in rest and light work (87 mmHg, range 79—96 mmHg). This value is lower than the reported threshold pO_2 for the chemoreceptors in animals and humans.

3. Breathing of 33 per cent oxygen diminished the hyperventilation of heavy work and increased the arterial pO_2 to 183 mmHg (range 161—190 mmHg). This value is well above the reported threshold pO_2 for the chemoreceptors.

4. Breathing of 100 per cent oxygen further decreased the hyperventilation and increased the arterial pO_2 to 663 mmHg (range 635—685 mmHg).

5. It is concluded that the hyperventilation in heavy work cannot be explained simply as the result of an arterial hypoxia. Earlier experiments with sudden changes from air breathing to oxygen breathing during work, and work experiments with about 12 per cent oxygen in the inspired air here presented, however, make the assumption probable that the chemoreceptor impulses elicited at the arterial pO_2 obtaining in the air experiments sensitize the respiratory centre towards the work stimulus. Under this assumption it is possible to explain the part of the heavy exercise hyperventilation which can be eliminated by increasing the arterial

pO_2 up to the threshold pO_2 of the chemoreceptors, as the result of a hypoxic chemoreceptor drive. Analogous with this sensitizing effect towards the work stimulus it has earlier been shown that the low oxygen stimulus also sensitizes the centre to the CO_2 stimulus, whereas the combined effect of the CO_2 and the work stimuli rather seems to be an additive one.

6. That part of the hyperventilation during heavy work which persists when the arterial pO_2 has been increased to the threshold of the chemoreceptors is assumed to be caused by anaerobic metabolites produced in the working muscles, and its elimination by a further increase in the alveolar pO_2, *e. g.* by changing from 33 to 100 per cent oxygen in the inspired air, is thought to be due to an effect on the metabolism of the working muscles of the increased amount of physically dissolved oxygen.

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